Identification of members of the apple endomicrobiome with the potential to protect against European canker

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Apple (*Malus domestica* Borkh.) is a widely cultivated and important economic fruit crop in New Zealand (NZ). European canker, caused by the fungal pathogen *Neonectria ditissima*, is one of the most significant diseases of apple. Application of chemical fungicides is the main control strategy for European canker, which can cause fungicide resistance in pathogens, environmental pollution and also chemical residues on fruit. Endophytes have drawn considerable attention as a novel source of biocontrol agents as effective isolates can also extensively colonise the plant host tissue. The aim of this study was to identify apple endophytes as biocontrol agents of European canker. It was achieved by i) identification of the effect of different factors on the complete endophyte community of apple shoots, ii) investigation of *in vitro* biocontrol activity of culturable endophytic bacteria and fungi against *N. ditissima* and their mode of action, and iii) determination of whether endophytic bacteria with *in vitro* biocontrol activity against *N. ditissima* could colonise and show activity against *N. ditissima* in apple shoots.

Denaturing gradient gel electrophoresis (DGGE) was used to characterise bacterial and fungal endophyte communities in apple leaves and/or stems with specific focus on the effect of different factors. This study used propidium monoazide (PMA) to enrich amplification of DNA from endophytes. The 2nd leaf, 3rd leaf, green stem and woody stem of 'Royal Gala' from three sites and 'Braeburn' from site 2 were analysed by DGGE and this showed that tissue type, cultivar and site were the main factors influencing bacterial and fungal endophyte communities, with the endophyte taxa in 'Royal Gala' more variable than that in 'Braeburn'. Bacterial and fungal endophyte communities in leaves often differed from those in stems in 'Royal Gala' and 'Braeburn'. Bacterial and fungal endophyte communities in woody stem were less influenced by season and not affected by region.

A representative collection of 1004 bacterial and 87 fungal endophytes were isolated from apple leaves and stems from nine sites in Nelson and Hawke's Bay in the spring main sampling and three sites in Nelson in the autumn main sampling, with a small proportion of those bacterial isolates

from samplings conducted in Lincoln University Research Orchard and Plant & Food Research Orchard in Hawke's Bay. A dual culture plating assay was used to test the inhibition effect of bacterial and fungal endophytes on mycelial growth of *N. ditissima,* with 18 bacterial and 17 fungal isolates found to be antagonistic to *N. ditissima*. Sixteen bacterial (9 *Bacillus* spp. and 7 *Pseudomonas* spp.) and 13 fungal isolates (3 *Chaetomium* sp., 5 *Epicoccum* sp., 3 *Biscogniauxia* sp., 1 *Penicillium* sp. and 1 *Dothideomycetes* sp.) were identified by rDNA sequencing and showed to be potential biocontrol agents due to their similarity to reported endophytes and/or known biocontrol agents, but not plant pathogens.

Mechanisms of action were investigated using a series of *in vitro* bioactivity assays for bacterial and fungal antagonism, and by detection of antibiotic encoding genes for bacteria. A cell-free culture filtrate assay showed two *Bacillus* isolates 42-1206(19)b and R1GS-12b and one *Pseudomonas* isolate 27-801(89)b inhibited mycelial growth of *N. ditissima* by producing diffusible antifungal compounds. Apart from *Bacillus* sp. 41-1182(4)b, *Chaetomium* sp. 2-57f and *Biscogniauxia* sp. 2-51f, all the bacterial and fungal isolates identified as potential biocontrol candidates produced siderophores. Bioactive volatile compounds were not observed for any of the bacterial or fungal isolates. Antibiotic encoding genes were detected in three *Bacillus* isolates (R1GS-12b and 42-1206(19)b were positive for *Bam C* and *ItuD*, and R3L-1b positive for *sfp*) showing their potential to synthesise antibiotics (bacillomycin D encoded by *Bam C*, iturin A encoded by *ItuD* or surfactin encoded by *sfp*) to suppress *N. ditissima,* but no antibiotic encoding genes were detected in the *Pseudomonas* isolates.

Spontaneous rifampicin resistant bacterial mutants (Rif^{125ppm}) were produced from six selected bacterial isolates and used as inocula to assess their colonisation in detached apple shoots. *Pseudomonas* isolates (20-579(18)$b^{125ppmRif+}$, 7-208(18)$b^{125ppmRif+}$ and 31b3$^{125ppmRif+}$) but not *Bacillus* isolates (42-1206(19)$b^{125ppmRif+}$, 21-606(28)$b^{125ppmRif+}$ and R3L-6$b^{125ppmRif+}$) showed persistent colonisation and spread in detached shoots. Mutant strains 20-579(18)$b^{125ppmRif+}$ and 7-208(18)$b^{125ppmRif+}$ had potential as wound protectants when they were inoculated 14 days before *N. ditissima* in attached apple shoots, as shown by the reduced recovery frequency of *N. ditissima* from the stem sections surrounding the wounded inoculation point. The approach taken in this study has promise to identify strains that may help protect apples against European canker.

Keywords: *Malus domestica* Borkh., *Neonectria ditissima*, endophyte community, PCR-DGGE, culturable endophytes, biological control.

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Chapter 1 General introduction

1.1 Apple industry in New Zealand

Apple (*Malus domestica* Borkh.) is a widely cultivated and significant economic fruit crop in New Zealand (NZ). NZ apples hold a high value in key markets around the world, due to fruit quality and new varieties that meet the constantly changing demands of consumers (Anon., No date-a). The NZ apple industry has been involved in developing and adopting the Integrated Fruit Production (IFP) programme since the 1990s. This has resulted in a significant change in the industry by increasing the replacement of chemicals with more environmentally friendly 'softer' pesticides for disease and insect control and changing from a calendar schedule of pesticide application to application based on monitoring systems and treatment thresholds (Walker et al., 2009). Moreover, the NZ apple industry introduced the Apple Futures programme from 2008 to 2010 to implement a low pesticide residue production system for NZ apple growers to target the European Union (EU) market (Walker et al., 2015). It aimed to reduce pesticide residues to ≤ 10% of the EU maximum residue level (MRL) with a maximum of three residues detected in any sample.

New Zealand's apple industry supplies 25% of the southern hemisphere's fresh apple exports. With the increased export to Asia and better access to markets, NZ apple sales are providing a significant economic benefit to the country. NZ apple export sales are expected to reach the target of $1 billion by 2022 (Morrison, 2014).

1.2 Apple growth

Apple growing is very dependent on climate conditions. The climate in New Zealand, with warm and dry summers and cool winters, is ideal for growing apples. Areas with an obvious winter period, providing at least 1200 h per year with temperatures under 7.2°C, and a dry, warm summer with high sunshine duration are optimal for apple growth (Anon., No date-b).

Apple planting materials are produced for out-planting by nurseries. This planting material generally consists of scions grafted onto rootstock which are sold as one-year old plants. The nurseries aim to produce disease-free and high quality branched one-year old apple trees (Tustin, 2006) for out-planting in orchards.

In New Zealand, the planting systems in apple orchards are being converted from intermediate density (650-900 trees/ha) to intensive density (1250-3000 trees/ha). Semi-dwarf and dwarf rootstocks are used to achieve the high density planting systems (Tustin, 2006). In these high

density planting systems, light is used effectively due to the increased light interception and distribution in the canopy (Khan et al., 1998). This results in fruit production from younger plants and higher yield and quality of fruit, thus allowing quick economic returns. Excessive vigour of vegetative growth in the high density planting systems generally happens after 4-6 years (Tustin, 2006) and can be overcome by management practices such as regulated deficit irrigation (Ebel et al., 1993) and root pruning (Khan et al., 1998).

In intensive planting systems, the tree canopy is controlled by using growing structures such as vertical axie or tall spindle style central leader trees planted in a row. In these systems, trees are supported by vertical wooden posts and wire trellis and grow into slim and well-spaced hedgerows with pendant side branches. Malling (M.) 9 rootstock is usually used for high density planting systems since it is reported to promote more pendant side branches than the other commonly used rootstock M.26 (Tustin, 2006). There are also other canopy shapes such as slender spindle, Y-trellis and V-trellis, with minor difference in light interception and fruit yield and quality among them (Hampson et al., 2002).

1.3 Apple diseases in New Zealand

Apple trees are susceptible to a wide variety of diseases caused by viruses, bacteria, fungi, mycoplasmas and nematodes. These lead to growth and yield reductions and can even result in the death of a tree (Ghasemkhani, 2012). Some of the important diseases of apple are fire blight, black spot, powdery mildew and European canker.

1.3.1 Fire blight

Fire blight, caused by *Erwinia amylovora*, is the most severe bacterial disease affecting apples in NZ orchards. *Erwinia amylovora* usually first attacks flowers in spring, resulting in brown colouration to the flowers and wilt of flower stems. Then, the infection moves to shoots and branches, with the typical symptoms of light tan droplets of bacterial ooze on the infected tissues (Ohlendorf, 1999). A single shoot infection can spread rapidly down through healthy branches, limbs and trunk and cause a girdling infection near the graft union of trees, resulting in the death of the whole tree (Johnson & Stockwell, 1998; Jones & Aldwinckle, 2003). The most commonly used apple rootstocks like M.9 and M.26 and the most commonly used commercial scion cultivars such as ‘Braeburn’, ‘Fuji’ and ‘Gala’ are highly susceptible to fire blight, causing significant financial losses for apple growers (Johnson & Stockwell, 1998; Jones & Aldwinckle, 2003). The pathogen spreads faster in vigorously growing trees which are under 8 years old than in older trees (over 20 years old) (Ohlendorf, 1999).

1.3.2 Black spot

Black spot (also called apple scab), caused by *Venturia inaequalis*, is one of the most widespread fungal diseases of apples. It causes more economic loss in temperate and humid regions than in semi-arid regions (Ghasemkhani, 2012). The first symptoms of black spot are lesions on leaves. Lesions on the upper leaves appear circular with clear margins and a black and scabby appearance, while lesions on the lower leaf surface shows irregular shape and velvety texture. As the fungus inhibits the leaf growth, leaves become twisted. Black spot can be seen on infected fruits, leading to a direct reduction in yield (Ohlendorf, 1999). Ascospores of *V. inaequalis* are the main source of primary inoculum at the onset of spring. After an ascospore has infected susceptible tissues such as green leaves and fruitlets and caused a lesion, the scab lesions can produce asexual conidial spores as the secondary infection. Spore infection is influenced by the number of hours of continuous leaf wetness and temperature in spring. When the temperature is 16-24°C, infection can happen within 6 hours of continuous leaf wetness. As the temperature decreases to 5°C, the leaf wetness period required for causing infection increases to around 20 hours (Manning, 2001).

1.3.3 Powdery mildew

Powdery mildew caused by *Podosphaera leucotricha* is another important fungal disease of apples in NZ. The pathogen *P. leucotricha* can survive in dormant flower and shoot buds overwinter, providing primary inoculum for the following year. It colonises the young, green shoots and leaves as they emerge from the bud in spring. On infected leaves, whitish patches commonly appear along the underside leaf margins. The powdery white areas then spread from the leaves to the shoots, resulting in the silver-grey ‘flag shoots’. The shoot may show defoliation, stunted growth, and dieback. As the plants grow, the primary infections on the flag shoots produce windborne spores which cause secondary infections on leaves, blossoms and fruit. The weakened infected trees have a higher risk of being invaded by secondary pathogens (Burchill, 1960; Ohlendorf, 1999). The quality of fruit is also downgraded because of the netlike russeting caused by the infection (Ohlendorf, 1999). Spores of *P. leucotricha* can germinate and cause infection under dry condition, which is different from fire blight and black spot. High temperature (over 35°C) usually inhibits the development of this disease (Ohlendorf, 1999).

1.4 European canker in apple

There are several different fungi, such as *Valsa mali*, *Botryosphaeria dothidea*, *B. obtusa* and *Neonectria ditissima*, that cause canker on apple trees (Amponsah et al., 2017b; Brown-Rytlewski & McManus, 2000; Ke et al., 2014). They can all reduce the growth and yield of apple trees and may cause the death of the whole tree. Of these, the leading cause of trunk disease of apples worldwide is European canker, caused by the fungus *N. ditissima* (Ghasemkhani, 2012).

European canker is prevalent in commercial apple orchards in most temperate growing regions of the world, although is not recorded in Australia (Anon., 2017; Langrell, 2002). In NZ, European canker is one of the most important diseases of apple, with epidemics reported to occur following wet growing seasons. The disease is now well established within the productive apple growing areas of Motueka and Riwaka. Due to the long latency of the pathogen in infected wood, removing inoculum from infected orchards is difficult. Further, surveillance of infected orchards and removal of infected wood is time consuming and onerous. The need to maintain tight fungicide control regimes throughout the season and to protect wounds increases crop management costs. Infection of major limbs and trunks results in the need for severe pruning and the removal of trees to remove inoculum, further reducing productivity. Furthermore, many of the modern varieties, such as 'Scifresh', 'Scilate' and 'Pacific Beauty', that are increasingly being planted, are relatively susceptible to the disease (Anon., No date-c).

1.4.1 Disease symptoms

The pathogen typically infects apple tissues through wounds produced by bud break, leaf fall, petal fall (fruit calyx), harvesting and pruning (Anon., No date-c). The first sign of infection is a blistering on the bark, sometimes with flaking bark, followed by the development of an elliptical canker that gradually increases in size as it shrinks away from the surrounding healthy tissue (Figure 1.1-a, b). In NZ, infections often occur during leaf fall being from May to June. However, the first symptoms are generally not seen until late August to October. Symptoms of infection of picking/harvesting wounds are often seen in April to August. For the winter pruning infection, the first visible signs appear in October or November, as flaking bark surrounding the wound (Figure 1.1-c). Flaky bark near the canker will also develop into cankers, resulting in the expansion of the canker lesion areas. Vascular staining can be found in the wood underneath the canker (Figure 1.1-d) (Anon., No date-c). These cankers eventually girdle the plant, leading to dieback and death of tissue. If they occur in the main trunk or the crutch of main branches, then significant portions of the plant are affected and might cause the loss of an entire tree (Anon., 2002). Cankers on trunks are serious because of the provision for both primary inoculum and entry points for more

infection. The pathogen can also infect fruit, resulting in eye rot in the orchard or storage rot after prolonged storage (Xu & Robinson, 2010). Distinguishing European canker from other cankers is significant for providing practical management for controlling this disease.

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Figure 1.1 Symptoms of European canker caused by *Neonectria ditissima* on (a) stem, (b) well developed European canker infection with concentric rings of cracking, (c) flaky bark and (d) vascular staining of the wood below the canker. Source http://hortinfo.co.nz/canker/#techbox =1&sm=0&sm1=1.

1.4.2 Pathogen

European canker is caused by the fungal pathogen *Neonectria ditissima* (formerly *Neonectria galligena*, anamorph *Cylindrocarpon heteronema*) (Castlebury et al., 2006; Ghasemkhani, 2012). *N. ditissima* belongs to the phylum Ascomycota, order Hypocreales, family Nectriaceae (Ghasemkhani, 2012). It has a wide host range and has been found on over 60 tree and shrub species from more than 20 genera, such as apple (*Malus* spp.), pear (*Pyrus* spp.) and beech (*Fagus* spp.) (Flack & Swinburne, 1977; Weber, 2014). Walter et al. (2015) reported that around 30% of shelter belt plant species found associated with NZ apple orchards were known to be European canker hosts or a member of a genus with a known host.

N. ditissima produces two types of spores. The first is the asexual conidium produced on the cankers when rains begin, especially during spring and autumn (Ohlendorf, 1999). They appear as white conidial sporodochia on the surface of the cankered bark (Figure 1.2-a) (Weber, 2014). Macroconidia are produced from multi-branched conidiophores. The macroconidia (4.5-5.5 × 52-62 µm) are cylindrical, straight or slightly curved, 1- to 7-septate and typically round ended.

Microconidia (2-3 × 4-8 µm) are cylindrical, aseptate, round ended. Microconidia are not produced from multi-branched conidiophores, but from the tips of small hyphal branches of the mycelium by abstraction (Sutton et al., 2014; Weber, 2014; Zeller, 1926b). The other type is the ascospore which is sexually produced in asci within small and red perithecia (ovoid-pyriform, 250-350 µm in diameter and 300-450 µm long) which are produced on the surface of old cankers in autumn and winter (Figure 1.2-b) (Ohlendorf, 1999; Sutton et al., 2014). Each ascus (stalk shaped, 12-15 × 75-95 µm) usually contain eight ascospores, which are hyaline, ellipsoidal or spindle shaped with one septum (Sutton et al., 2014).

Macroconidia were found to be highly infectious, while the role of microconidia in the epidemiology of the disease is uncertain (Weber, 2014). However, their function is thought to be similar as microconidia germinate on agar media in a similar way to macroconidia. Both macroconidia and microconidia can be dispersed by rain-splash, whilst ascospores of *N. ditissima*, once released from the perithecial ostiole, are dispersed by wind (Weber, 2014).

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Figure 1.2 *Neonectria ditissima* (a) conidia (white spores) produced in the concentric cracks during humid weather, especially in spring and autumn and (b) perithecia, seen as dark red fruiting bodies produced in autumn and winter. Source http://hortinfo.co.nz/canker/#techbox =1&sm=0&sm1=1.

1.4.3 Disease cycle

The disease cycle of European canker starts with the production of spores, including both conidia and ascospores (Figure 1.3). In spring, when the temperature is above freezing and moisture levels are high, mainly conidia are produced on lesions and dispersed by rain. The main peak of conidia production occurs from around flowering time onwards (Weber, 2014). The conidia can be splashed up to a 3 metre distance from the tree and 1 metre up the tree canopy (Anon., No date-c). Leaf scars, caused by leaf fall, are most susceptible to infection up to an hour after being formed. However, the leaf scars remain susceptible up to 28 days after they are produced (Ohlendorf, 1999). The conidia can also infect mechanical wounds produced by weather damage,

wind, or pruning (Weber, 2014). After the infection occurs, lesions become visible 2 to 3 months later (Ohlendorf, 1999). In late summer and autumn when the weather is wet, perithecia are predominately produced on old cankers that have been initiated by infections more than 8 months previously (Anon., No date-c; Weber, 2014). Ascospores are released after the perithecia have matured and have been found to only be discharged when the leaves are wet. Rainfall of over 1 mm is sufficient for allowing spore release (Anon., No date-c; Zeller, 1926b), with the high humidity conditions after rain being favourable for ascospore release (Swinburne, 1971). The ascospores are released mostly in late summer and autumn, but can also be released at other times of the year. The liberated ascospores are then dispersed over hundreds of metres to up to a few kilometres through air currents and therefore potentially resulting in initiation of new infections at some distance from the disease origin (Agrios, 1997). *N. ditissima* survives either as mycelium within infected tissue or in the visible perithecia through the winter and under adverse environmental conditions (Ghasemkhani, 2012).

Conidia and ascospores are both produced throughout the year whenever rainfall occurs. Amponsah et al. (2017b) reported more spores were trapped under frequent rainfall conditions. The production of both types of spores reach a peak at 10-16°C in autumn (Ghasemkhani, 2012). A consensus emerging from the observations in New Zealand is that inoculum availability of both types of spores peaks in April-May (Amponsah et al., 2017b).

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Figure 1.3 Disease cycle of *Neonectria ditissima* (Agrios, 1997).

1.4.4 Infection

1.4.4.1 Infection conditions

N. ditissima requires a wound to penetrate into plant tissues to cause infection. There are many types of wounds available for infection during the year. Autumn leaf scars caused by leaf fall are found to be one of the most significant entry sites for *N. ditissima* (Gaskin et al., 2014). In addition, wounds caused by pruning, thinning, harvesting, bark cracks caused by frost injury or trunk expansion, or wounds caused by insects, other pathogens or hail damage also provide various entry points for *N. ditissima* during the whole year (Weber, 2014).

Peak infection may occur in the autumn at leaf fall because of the formation of a large number of leaf scar wounds and the availability of the largest number of both ascospores and conidia during this time (Sutton et al., 2014). Temperature and leaf wetness periods are two important factors influencing leaf scar infection, because of their effect on conidial and ascospore germination. Conidial germination rates increase as the temperature increases from 6°C to an optimum at 20-25°C (Weber, 2014). As the temperature increases from 10°C to 20°C, the minimum leaf surface wetness period required for leaf scar infections is reduced from 24 h to 2 h (Latorre et al., 2002). Ascospore germination was reported to be more influenced by temperature than conidial germination. Germination of ascospores increased 2.6 times faster than conidia when the temperature was increased from 5°C to 20°C (Latorre et al., 2002).

Wound age is another factor affecting wound infection. Older wounds are generally less susceptible to infection probably as a result of wound healing caused by the physiological activity of the plant. Wound infection will fail if the wound infection process is longer than the wound healing process (Weber, 2014). Amponsah et al. (2017a) found wounds on older wood (3-year old) developed more and longer lesions than on younger wood (1 or 2-year old). This could be because wounds on younger wood are quicker to be healed than those on old wood. Lesion development on artificial wounds was neither affected by the dates under different weather conditions nor the timing of the inoculation day (morning vs. evening) (Patrick et al., 2018).

Inoculum dose is also an important parameter for successful wound infection. This is because an inoculum mass is required to overcome plant defence mechanisms such as the production of callus barriers produced by the host around the canker (Weber, 2014). Dubin and English (1974) reported that the inoculum concentration of 5×10^1 and 5×10^3 macroconidia/leaf scar caused 20% and 90% leaf scar infection under natural conditions, respectively. Walter et al. (2016) showed that 10 to 30 conidia could initiate infection on freshly-made pruning or rasp wounds in

the field. Further, it was also suggested that different inoculum concentrations are required for successful infection of different wound types.

1.4.4.2 Infection process

The fungal hyphae produced by the germinating spores initially invades the xylem fibres and then stays dormant in the xylem (Weber, 2014). The pathogen also penetrates the soft tissues outside of the xylem. When the pathogen penetrates the soft peripheral tissue, a phellogen barrier is formed. This results in the blockage of the vascular tissue, which temporarily prevents further damage to the plant (Clatterbuck, 2006). However, the barrier can be broken by accumulation of mycelium near the barrier due to either mechanical pressure or the increased concentration of toxins produced by the accumulated mycelium. In response, a new phellogen is formed in the soft tissue outside the infection site to repeat the process (Ghasemkhani, 2012). As a result, the pathogen is allowed to spread in the xylem fibre and lesion size expands.

In the process of phloem infection, phloem cells are killed by toxins produced by the pathogen. The fungal hyphae then penetrates the dead phloem fibre cells and causes infection (Ghasemkhani et al., 2016; Mišíková et al., 2018). Due to the reaction of the host to infection, phellogen is also formed by the host at the margin of the infection site. With the breakage of the phellogen by the accumulated hyphae and new formation of phellogen by the host, the pathogen spreads in the form of mycelium in the phloem fibre and expands the lesion. The mycelium first grows within the phloem fibre cells and then develops intercellularly into surrounding fibre (Ghasemkhani, 2012).

The fungus also enters the host tissue through small cracks in the leaf scar tissue to cause infection at the margin of the leaf scar, resulting in reddish brown spots as the sign of infection (Campos et al., 2017). After the pathogen enters into the stem, it grows intercellularly within the tissues around the leaf base which are looser than other parts of the cortex. The hyphae then spreads to other cortical tissues, resulting in the formation of mycelial strands (Ghasemkhani et al., 2016). Then, the entire stem can be girdled by the pathogen. A phellogen barrier is also produced by the host close to the infected area and then broken down by the pathogen, resulting in the expansion of the infection site. Once buds are infected, the canker will result in the shoots above the infection site dying. When the fungus enters into the host through other wound sites such as those caused by frost injuries, the spores germinate in the wound sites and the pathogen then destroys the cortical tissues of the bark. As a consequence, more infection can be caused through the damaged bark (Ghasemkhani, 2012).

1.4.5 Climate conditions for disease development

The occurrence of European canker depends on rainfall and temperature conditions. Countries with a high risk of European canker, including the United States, Chile and the United Kingdom, have climates with both rain occurring on more than 30% of days per month and an average of over 8 h per day with a temperature of between 11 and 16°C (Beresford & Kim, 2011). These two climate conditions were defined by Beresford and Kim (2011) as the threshold to classify the disease risk of a region. Based on these thresholds, Auckland, Nelson and Hawke's Bay are all considered to be favourable regions for European canker development with different levels of risk. Auckland was found to be highly conducive to European canker because both the rainfall frequency and the temperature range thresholds were exceeded from April to November. In comparison, the Nelson region was reported to be less favourable for European canker, with both the rainfall frequency and temperature thresholds being exceeded from April to May and from September to November. Weather conditions in Napier, Hawke's Bay are the least favourable of these regions for European canker development, with both rainfall and temperature thresholds only being exceeded to a limited degree between April and November (Beresford & Kim, 2011).

When the monthly precipitation is over 100 mm, European canker risk for that month mainly depends on the amount of rainfall but not rainfall frequency. Therefore, rainfall amount should also be considered in order to prevent the underestimation of risk prediction when rainfall frequency does not exceed the threshold (Amponsah et al., 2017b; Kim & Beresford, 2012).

1.4.6 Control strategies for European canker in apple

1.4.6.1 Field inspection

Regular monitoring of the orchard is recommended all year round. Inspection of the orchard for European canker is carried out when the sun is high as this can reduce the risk of not seeing cankers. As cankers are often hidden on the lower sides of branches, trees are recommended to be inspected starting from the lower parts of the tree moving to higher parts (Anon., No date-c). It is important that all orchardists should manage this disease in their orchards, preventing the production of inoculum to infect neighbouring orchards.

1.4.6.2 Cultural control strategies

Cankers on infected trees are the main inoculum source in an orchard (Anon., No date-c). Cutting out infected branches and removing any visible canker lesions are key ways to curb the epidemic (Ghasemkhani, 2012). As latent periods can last from 2 months to 3 years, lesion removal is carried out all year round. Furthermore, the excised infected tissues is burned after removal

(Anon., No date-c), as ascospores can be released from this tissue for up to two years (Weber, 2014). Additionally, European cankers on young branches and side shoots can be removed by pruning. Pruning is commonly done in winter and summer in NZ orchards. Healing of the pruning wound is slower in winter than during the vegetation period, mainly because of the lower plant physiological activity under low temperatures (Weber, 2014).

As *N. ditissima* is a wound parasite, a pruning paste is needed to be used for covering pruning wounds immediately after pruning (Anon., No date-c). However, removing canker lesions is both time consuming and results in high labour costs (Gustavsson, No date). As *N. ditissima* also can infect a wide range of broad-leaved trees such as pear (*Pyrus* spp.) and alder (*Alnus* spp.), any cankers which develop on neighbouring pear orchards (Anon., No date-c) or shelter belt trees such as Italian alder (*Alnus cordata*), black alder (*Alnus glutinosa*) and the NZ native kowhai (*Sophora microphylla*) (Walter et al., 2015) also need to be removed. Sanitation practices are recommended in the orchard. It is important to remove pruning, fruit cropping or harvesting debris from the orchard as these can act as inoculum sources.

In addition, vertical shelters are recommended to be built between low risk blocks and neighboring blocks with high risk in order to prevent the contamination of the low risk blocks (Anon., No date-c). Since young nursery trees can also be infected by *N. ditissima* (Figure 1.4), it is also essential to select healthy trees without lesions as planting material for the orchard (Anon., No date-c).

Figure 1.4 Young tree girdled by European canker lesion caused by *Neonectria ditissima*. Source http://hortinfo.co.nz/canker/#techbox=2&sm=0&sm1=3&sm2=1.

1.4.6.3 Breeding of resistant cultivars

Most apple cultivars commonly planted in commercial orchards, such as 'Pacific Queen' , 'Scifresh', 'Scilate' and 'Royal Gala', are susceptible to European canker (Anon., No date-c). Breeding cultivars with high level of resistance to European canker is considered to be an effective way to control this disease (Gelvonauskienė et al., 2007). Although complete resistance has not yet been identified in apple, different levels of partial resistance has been reported which can provide valuable resource material for plant breeding programmes (Ghasemkhani et al., 2015). Molecular markers used in breeding make the breeding programs faster by focusing on specific genes linked to desirable traits among related species. *N. ditissima* resistance locus *Rnd1* has been identified from *Malus* 'Robusta 5', which is highly resistant to European canker (Bus et al., 2015; Bus et al., 2019).

1.4.6.4 Chemical control strategies

Fungicides play an important role in reducing infection of apples by *N. ditissima*. In NZ, there are several crucial periods for fungicide application. During leaf fall in autumn, fungicides such as Captan, EuroGel™ (containing boric acid and octhilinone), Bordeaux mixture (containing copper sulfate and slaked lime), and copper oxychloride are commonly used. Bordeaux mixture serves as an effective fungicide because it can be redistributed by rainfall. Slaked lime has also been tested for controlling European canker in the Nelson region of NZ and was found to be effective against European canker. Carbendazim also plays an important role in preventing wound infection at the beginning of leaf fall. Due to the potential environmental toxicity of copper formulated fungicides, the rate of copper oxychloride in one application is limited at 4.3kg/ha in NZ. During the period from 10% to 90% leaf fall, Captan, and EuroGel™ are recommended to be sprayed weekly (Anon., No date-c; Walter et al., 2019). In order to reduce the number of fungicide sprays, EDTA Copper applied at the rate of 1 kg Cu/ha is commonly used to speed up leaf fall in New Zealand (Anon., No date-c).

Another important time for applying fungicides is after winter pruning. Wounds produced by pruning can be protected from infection by painting with pruning gel containing carbendazim or with EuroGel™. During the spring-summer period, fungicide application is a significant way to control European canker infection of side shoots or small branches by inhibiting the production of ascospores and conidia of the pathogen (Swinburne, 1975). During the spring-summer bud break period, dodine is effectively used for protecting against apple black spot but it has no effect on European canker. Therefore, dodine is commonly mixed with captan or carbendazim to protect wounds produced by bud-break from *N. ditissima* infection (Anon., No date-c). Summer pruning

wounds are usually protected as for winter pruning wounds using a pruning gel. Moreover, trees can also be protected by application of captan and dithianon (Cooke, 1999; Swinburne, 1975) which are used for apple black spot control during this time (Anon., No date-c; Weber, 2014). In New Zealand, coppers, captan, carbendazim and slaked lime are currently four main groups of sprayable chemicals for European Canker protection (Anon., No date-c). Based on NZ Novachem Agrichemical Manual (Anon., 2018/2019), only copper related products including AG Copp 75, Copper Oxychloride and Cuprofix Disperss are registered for European canker control. Captan, carbendazim and dithianon were registered for other pip fruit fungal diseases such as black spot.

Although fungicides play an important role in reducing the disease development, they cannot eliminate the fungus within established infections. Additionally, protectant fungicides may not provide long-term protection of wounds and may require frequent application. For example, more leaf scars can be produced after a rain event or storm because of the increased leaf drop (Gaskin et al., 2014). Adjuvants are often used since they are reported to improve the effect of fungicide spray by promoting deposition, rain fastness, and rain redistribution (Gaskin & Steele, 2009; van Zyl et al., 2010). Gaskin et al. (2014) reported the use of organosilicone/latex sticker adjuvants increased captan retention on fresh leaf scars, and superspreader-type adjuvants (Bond Xtra, Du-Wett WeatherMax and Gro-Wet) increased captan retention on stem wood and leaf nodes due to the rain redistribution from leaves. However, additional fungicide sprays are required to cover the newly produced leaf scars to provide sufficient protection of all the wounds. The use of fungicides, however, are costly and their frequent use increases the risk of fungicide resistance developing in *N. ditissima* (Weber & Palm, 2010). This has recently been seen in the NZ apple industry for another pathogen with the observation of *V. inequalis* populations having increasing resistance to demethylation inhibitor (DMI) fungicides (Beresford et al., 2013).

Chemical fungicides are still the major and indispensable strategy for controlling European canker in apple, as there have been limited reports of any biocontrol agent or commercial biological product effective at controlling this disease. Walter et al. (2017b) tested biological products including those containing *Bacillus subtilis*, *Pseudomonas putida*, *Trichoderma koningii* and *T. harzianum* either alone or combination, and reported none provided adequate wound protection when challenge-inoculated with at least 10^3 *N. ditissima* spores/wound.

1.5 Biological control

1.5.1 Biological control of plant pathogens

Biocontrol strategies have drawn much attention as substitutes for chemical control. Biocontrol measures are based on the use of antagonistic organisms against the target pathogens (Benítez et al., 2004). Biological control includes the use of both the beneficial organisms and/or metabolites produced by them to reduce the negative effects of plant pathogens and promote positive responses by the plant (Vinale et al., 2008). Most plants show symbiotic relationships with rhizobacteria, mycorrhizae and endophytes (Bloemberg & Lugtenberg, 2001; Petrini, 1986). Rhizobacteria and mycorrhizae colonise plant roots and grow into the surrounding soil, while endophytes live entirely inside plant tissues and are able to grow within roots, stems and leaves. Some of them play an important role in plant ecology, fitness and evolution (Brundrett, 2006; Carroll, 1988; Nadeem et al., 2014).

Some rhizobacteria have beneficial influence on the plant hosts such as suppression of soil-borne diseases and stimulation of plant growth (Bloemberg & Lugtenberg, 2001). They play a significant role in biocontrol studies. For example, *Pseudomonas*, *Bacillus* and *Streptomyces* species were known to be biocontrol agents of various soilborne pathogens such as *Rhizoctonia solani* in wheat (*Triticum aestivum*) (Gull & Hafeez, 2012), *Rosellinia necatrix* in avocado (*Persea americana*) (Cazorla et al., 2007) and *Ralstonia solanacearum* in tomato (*Solanum lycopersicum*) (Lemessa & Zeller, 2007), respectively.

Mycorrhizae including both arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (ECM) have been shown to have biocontrol activity against soilborne pathogens of various plants. For example, AMF were reported to reduce disease incidence caused by *Fusarium oxysporum* f. sp. *asparagi* in asparagus (*Asparagus officinalis*), *Phytophthora capsici* in pepper (*Capsicum annuum*) and *Fusarium oxysporum* f. sp. *lycopersici* in tomato (*Lycopersicon esculentum*) (Bidellaoui et al., 2019; Matsubara et al., 2001; Ozgonen & Erkilic, 2007). Ectomycorrhizal fungi have also been shown to reduce disease severity of *Fusarium* damping off in pine (*Pinus* spp.) seedlings (Chakravarty & Hwang, 1991; Mateos et al., 2017) and *Phytophthora* ink disease in chestnut (*Castanea sativa*) (Blom et al., 2009; Branzanti et al., 1999; Scattolin et al., 2012).

Endophytes refer to bacteria and fungi growing within plant tissues without causing apparent disease symptoms (Hallmann et al., 1997; Petrini, 1986). All plant species are colonised by a community of endophytes (Porras-Alfaro & Bayman, 2011). This group of microbes may be considered as the second genome or the pan-genome for the plant host (Turner et al., 2013). It is well established that their interaction with plants can provide many beneficial responses such as

improved growth, disease resistance and resistance to abiotic stress (Afandhi et al., 2019; Bae et al., 2011; Gupta et al., 2012; Porras-Alfaro & Bayman, 2011; Rajkumar et al., 2009; Ripa et al., 2019; Tran et al., 2019). They are targeted as an important source of potential biocontrol agents. For example, endophytic bacteria such as *Bacillus* spp., *Enterobacter* spp., *Lysobacter* spp., *Pantoea* spp., *Pseudomonas* spp. and *Streptomyces* spp. and endophytic fungi like *Aspergillus* spp., *Gliocladium* spp., non-pathogenic *Fusarium* spp., *Petriella* spp. and *Trichoderma* spp. have been found to be important biocontrol agents (De Silva et al., 2019).

In addition, mycoviruses, viruses that infect fungi, are widespread throughout the main fungal genera. Their properties in reducing the ability of their fungal hosts to cause disease in plants, known as hypovirulence, made a significant contribution to biocontrol studies (Hammond et al., 2008; Schmitt & Breinig, 2006; Strauss et al., 2000; Yu et al., 2013; Zheng et al., 2019; Zhu et al., 2018). For example, fungal viruses in the family Hypoviridae play a critical role in the control of the chestnut blight fungus, *Cryphonectria parasitica,* through hypovirulence (Milgroom & Cortesi, 2004). Biocontrol of chestnut blight by mycoviruses is considered as a model in tree disease management (Muñoz-Adalia et al., 2016).

1.5.2 Biological control of apple diseases

Biological control of apple diseases has drawn increasing attention as a means of reducing the use of chemical fungicides. Reducing the environmental impact of chemicals and chemical residuals in fruits provides a sustainable apple production system.

Arbuscular mycorrhizal fungi (AMF) found to colonise apple roots were shown to have a biocontrol effect on reducing the severity of Botryosphaeria canker of apple, caused by *Dematophora necatrix* of apple saplings, reduction in soil nematode damage of apple seedlings and growth promotion of apple plants under glasshouse conditions (Ceustermans et al., 2018; Krishna et al., 2010; Raj & Sharma, 2009). More importantly, AMF colonisation was reported to increase the resistance of grafted apple trees to *N. ditissima*, with a reduction in the number of infected materials by an average of 18% (Berdeni et al., 2018).

The endophytic fungus, *Chaetomium spirale* ND35 isolated from *Populus tomentosa* was show to colonize the stems and branches of apple trees. The fungus was reported to be an effective biocontrol agent for apple canker caused by *Valsa ceratosperma* both in greenhouse and field trials. *C. spirale* ND35 was shown to inhibit mycelial growth of *Vsa. ceratosperma* by producing antibiotic substances. Through the production of cell wall degrading enzymes such as chitinases and β-glucinases, *C. spirale* ND35 is able to mycoparasitise the hyphae of *V. ceratosperma* (Xin & Shang, 2005). *C. spirale* ND35 was also shown to inhibit *Rhizoctonia solani* by mycoparasitism

which was mediated by an amorphous matrix enriched with β-1, 3-glucan formed from the cell wall of *C. spirale*. The β-1, 3-glucan-enriched matrix then sticks to the surface of *R. solani* hyphae to induce the formation of entry sites for *C. spirale* ND35 (Gao et al., 2005). However, there have been no studies on the identification of apple endophytes antagonistic to *N. ditissima*.

1.6 Endophytes

1.6.1 Ecology of endophytes

Bacterial endophytes are found to colonise various tissues of a wide variety of plant species including monocotyledonous and dicotyledonous plants (Lodewyckx et al., 2002). It has been found that many bacterial endophytes initiate their colonisation of various plant organs from the root zone (Chi et al., 2005; Frank et al., 2017). The bacteria colonising the root surface and the rhizosphere can break the endodermis barrier and enter the vascular system by crossing from the root cortex, and then colonise as endophytes in other organs such as the stems and leaves (Compant et al., 2005). In addition, Compant et al. (2005) suggested that colonisation by some bacterial endophytes could originate from bacteria colonising the surface of other organs such as the phyllosphere, anthosphere and spermosphere. Bacterial endophytes were also proved to be able to enter plants through pruning wounds (West et al., 2010).

Endophytic fungi are commonly divided into clavicipitaceous endophytes (C-endophytes) and nonclavicipitaceous endophytes (NC-endophytes) based on fungal phylogeny, their colonisation traits and ecological function (Rodriguez et al., 2009). Until recently, most researchers have focused on C-endophytes due to their agricultural importance. For example, C-endophytes impact host resistance to insect feeding due to the effects of the fungal metabolites that they produce (Tanaka et al., 2005). However, the genetic diversity of C-endophytes is low. They include a small number of phylogenetically related clavicipitaceous species that are difficult to isolate and are present only in some cool- and warm-season grasses (Clay, 1989; Rodriguez et al., 2009). NC-endophytes are highly diverse and have been further classified into three different functional groups (Class 2, Class 3, and Class 4) based on their life history characteristics and ecological significance (Rodriguez et al., 2009). Of these, the Class 2 NC fungal endophytes have attracted more research attention than the Class 3 and the Class 4 NC fungal endophytes. This is because they can inhabit a wider range of plant tissue, including shoots, roots and rhizomes, compared with the Class 3 NC endophytes which only colonize shoots and the Class 4 NC endophytes which only colonize roots. Moreover, the fitness benefits of the Class 2 NC fungal endophytes include both nonhabitat-adapted benefits such as drought tolerance and growth enhancement regardless of the habitat of origin, and habitat-adapted benefits related to habitat-specific selective

pressures such as pH and salinity (Rodriguez et al., 2009). Furthermore, many Class 2 NC endophytes increase the disease resistance of plants due to the production of secondary metabolites (Schulz et al., 1999), fungal parasitism (Samuels et al., 2000), or induction of systemic resistance (Vu et al., 2006).

1.6.2 Inhibition of plant pathogens by endophytic microbial community

Members of the endophyte communities are increasingly being targeted as a source of potential biocontrol agents for plant diseases (Backman & Sikora, 2008; Eljounaidi et al., 2016; Lodewyckx et al., 2002). For example, soybean bacterial endophytes *Bacillus* sp. and *Burkholderia* sp. significantly inhibited the *in vitro* growth of the fungal pathogens *Sclerotinia sclerotiorum*, *Phomopsis sojae* and *Rhizoctonia solani* (de Almeida Lopes et al., 2018). Bacteria belonging to the gammaproteobacteria such as *Pseudomonas* spp. (Bakker et al., 1990; Wicaksono et al., 2016; Zhou & Paulitz, 1994) and *Enterobacter* spp. (de Almeida Lopes et al., 2018; Taghavi et al., 2009), and actinobacteria such as *Streptomyces* spp. are also important groups of endophytic bacteria which have been studied as potential biocontrol agents (Cao et al., 2016; Vurukonda et al., 2018). Endophytic fungi such as *Chaetomium globosum*, *Epicoccum nigrum* and *Trichoderma* spp. have been widely studied as potential biocontrol agents against various fungal pathogens in plants (Cullen et al., 1984; Hanada et al., 2010; Hashem & Ali, 2004; Kortekamp, 2015; Lahlali & Hijri, 2010; Soytong et al., 2001). For woody plants, endophytes play an important role as antagonists of pathogens. A strain of *Bacillus subtilis* from grapevine wood showed strong *in vitro* inhibition effect on mycelial growth and ascospore germination of the fungal pathogen *Eutypa lata* causing dieback in grapevines, and also significantly reduced *E. lata* infection of pruning wounds under field conditions (Ferreira et al., 1991). Similar findings were also reported for other pathogens of woody plants such as *Candidatus Liberibacter asiaticus* and *Phyllosticta citricarpa* in Citrus (*Citrus* spp.) (Tran et al., 2019; Trivedi et al., 2011) and *Ceratocystis fagacearum* in oak (*Quercus* spp.) (Brooks et al., 1994). Therefore, members of the endophytic microbial communities have potential to inhibit pathogen infection, with disease suppression potentially dependent on the endophyte community composition in plants.

The endophyte communities have been shown to be affected by cultivars (Andreote et al., 2010; Jayawardena et al., 2018) and therefore could contribute to the relative susceptibility of plant cultivars to particular pathogens. A higher frequency of strains with antagonistic activity against the grapevine fungal pathogen *Botrytis cinerea* was obtained from a resistant variety (Solaris) than from susceptible varieties (Chasselas and Pinot noir) (Vionnet et al., 2018). Hirakue and

Sugiyama (2018) found the lower apple leaf damage caused by *Alternaria mali* in a resistant apple cultivar was closely related to the total abundance of fungal endophytes in leaves.

Endophyte community has also been correlated to plant health status (diseased or healthy). Köberl et al. (2017) identified members of gammaproteobacteria as potential health indicators of banana plants in *Fusarium* wilt-infested fields, with populations of plant-beneficial *Pseudomonas* and *Stenotrophomonas* increased in healthy plants and *Enterobacteriaceae* in diseased plants. Similarly, ectomycorrhizal communities were shown to be an indicator between healthy and ink disease-infected sweet chestnut trees (*Castanea sativa*) (Blom et al., 2009; Scattolin et al., 2012).

Endophyte community have also been reported to be affected by soil conditions. The proportion of antagonistic bacterial isolates in the endophyte communities of pepper plants (*Capsicum annuum*) were shown to be affected when cultivated in different soils collected from different geographic locations in Korea (Kang et al., 2016). It is because above-ground plant tissues acquire microbiomes migrated from roots which were affected by soil condition (Chi et al., 2005). Determining the different factors which affect the endophyte community is therefore important when investigating endophytes as potential biocontrol agents.

1.6.3 Mechanisms of action as biocontrol agents

Biocontrol mechanisms of endophytes are classified into four categories, antibiosis, competition, host-induced resistance and direct parasitism (Card et al., 2016).

The mechanism by which bacterial endophytes have beneficial effects on the host plant is considered to be similar to that provided by plant growth-promoting rhizobacteria (PGPR) (Höflich et al., 1994; Zablotowicz et al., 1991). They can reduce the adverse influence of one or more plant pathogens on plant growth, thus suppressing disease development (Lodewyckx et al., 2002). One mechanism by which PGPR suppress disease is by inhibiting the multiplication of phytopathogens by producing siderophores with high iron affinities (Castignetti & Smarrelli, 1986). The proliferation of pathogens nearby is inhibited due to iron deficiency (Duijff et al., 1993). However, the growth of the plant is generally not affected by the iron deficiency since many plants are capable of binding the iron-siderophore complex with the iron then released when the iron-siderophore complex is transported inside the plant (Jin et al., 2006). In addition, PGPR are also found to suppress plant diseases through the production of antibiotics or enzymes, enhancing plant competition for nutrients and/or occupation on the root surface and inducing the systemic resistance of the plant (Bangera & Thomashow, 1996; Fenton et al., 1992; Lodewyckx et al., 2002). The PGPR *Enterobacter asburiae* BQ9 was shown to promote tomato plant growth and induce resistance to tomato yellow leaf curl virus (TYLCV) under greenhouse

conditions by increasing the expression of defense-related genes and H_2O_2 production (Li et al., 2016). Production of siderophores and antimicrobial compounds, and induction of plant resistance have been commonly used as biological indicators for identifying bacterial endophytes as potential biocontrol agents (Chen et al., 2019; Gond et al., 2015; Pliego et al., 2011; Trotel-Aziz et al., 2008).

Many endophytic fungi belonging to Class 2 NC endophytes have been shown to protect plants from pathogen infection due to the production of secondary metabolites (Arora & Kaur, 2019; Nisa et al., 2015; Schulz et al., 1999), fungal parasitism (Samuels et al., 2000), or induction of systemic resistance (Nassimi & Taheri, 2017; Vu et al., 2006). Rodriguez et al. (2009) suggested that the increased disease resistance of plants might also be due to the endophytes being better competitors for resources or space than fungal pathogens. Systemic growth of endophytic fungi in woody plants is often limited (Saikkonen, 2007). Yan et al. (2015) proposed that the antagonistic effects of fungal endophytes on phytopathogens are likely to be attributed to the metabolites they produced, rather than the systemic growth within plants.

1.6.4 Identification of the diversity of endophytes

Endophyte microbial community composition can be described by cultivation-dependent (culture-based methods) and independent methods (molecular methods).

1.6.4.1 Culture-based methods

Culture-based methods are traditional methods used to evaluate the diversity of culturable bacteria and fungi, based on the morphological characters of colonies or spores in culture. Fungal species can be distinguished by colony morphology such as colony pigmentation, growth rate, basic shape of the colony, elevation viewing from side of a colony, margin, surface texture, opacity and size of the colony (Anon., No date-d; Breakwell et al., 2007). Fungi are also differentiated by their spore morphology including colour, septation, and the way they are produced. Additionally, spore bearing structures such as perithecia, cleistothecia, pycnidia and apothecia are also widely used to identify fungi (Kurup et al., 2000). However, bacterial species cannot be differentiated by their colony morphology since many bacteria from different genera have similar colony morphologies.

Culture-based methods create a bias in the estimation of an endophytic community. The majority of endophytes are unculturable and therefore not available for functional assays (Tejesvi et al., 2016). This low percentage of culturable microbes is partly because of restrictions due to the media and conditions used for culturing (Venkatachalam et al., 2015). A large percentage of microbes have not been cultured because the conditions required for their growth are unknown

(Muyzer & Smalla, 1998; Torsvik et al., 1990). Moreover, the culturable endophytic bacterial population is also affected by the surface sterilisation method used. A surface sterilisation method that can completely kill surface bacteria can also kill internally colonised microbes to a certain extent (Lodewyckx et al., 2002). Therefore, species that cannot grow or grow slowly in culture media are often ignored and species that grow well in culture provide an over-representation of an endophytic community. In addition, sampling methods such as sample size including the number of plants sampled and number of samples per plant, types of tissues sampled and sample collection time all impact the diversity of endophytes isolated (Bayman, 2006; Gamboa et al., 2002). Therefore, a combination of a culturing method and a molecular method which is culture-independent is commonly used to investigate endophytic community of environmental samples (Ferrando et al., 2012; Jackson et al., 2013).

1.6.4.2 Molecular methods

Molecular techniques play a significant role in revealing taxa richness of microbial communities because of their advantage of being culture-independent (Giraffa & Neviani, 2001; Handelsman et al., 1998; Torsvik et al., 1990). Without the limitation of culturing, a more comprehensive assessment of the microbial community structure can be conducted.

16S ribosomal RNA (~1.5 kb) is the official barcoding region for bacterial classification. The 16S rRNA is generally conserved in all bacteria and also contains interspecies variability. It contains both conserved regions and nine hypervariable regions (V1-V9) with the length spanning between 50 and 200 bases (Clarridge, 2004; Forde & O'toole, 2013). It is most widely used for bacteria identification and phylogenetic trees (Bahadir et al., 2018; Etesami & Alikhani, 2018).

The internally transcribed spacers (ITS) of the ribosomal RNA (rRNA) gene region, 18S rRNA, β-tubulin and translation elongation factor 1-α [EF1- α] are commonly used as taxonomic genes for fungal identification (Denman et al., 2000; Liu et al., 2015; Slippers et al., 2004). A more conserved region of the ribosomal gene such as the SSU (small subunit) rDNA gene is more suitable for revealing fungal communities with many different species because they show less within-species variation compared to ITS sequences (Vainio & Hantula, 2000).

Endophytic diversity in an environmental sample can be analysed by environmental PCR using surface-sterilised plant materials. The diversity of endophytes can be revealed by the sequences of taxonomic genes and by genetic fingerprinting techniques through amplification of taxonomic genes using metagenomics DNA extracted from environmental samples (Maropola et al., 2015; Stackebrandt & Goebel, 1994).

Genetic fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE)/ thermal gradient gel electrophoresis (TGGE) are commonly used for studying the diversity of microbial communities in microbial ecology (Lodewyckx et al., 2002). First, taxonomic genes from the microbial community matrix are amplified by PCR. One of the primers often contains a GC-clamp to facilitate separation of PCR amplicons in a DGGE gel (Temmerman et al., 2003). The amplified DNA fragments with different sequences can then be separated in polyacrylamide gels with a linear gradient of DNA denaturants (DGGE) or a linear temperature gradient (TGGE). Taxonomic differences indicating microbial diversity can be shown by the band patterns in the gel, and the microbes of interest can be identified by comparing sequences of excised DNA fragments to reference sequences reported in nucleotide databases (Garbeva et al., 2001; Giraffa & Neviani, 2001; Ho et al., 2015). For example, Duong et al. (2006) effectively used PCR-DGGE with sequence analysis of 18S rRNA gene to assess the diversity of endophytic fungi in leaves of *Magnolia liliifera* and revealed the different fungal taxa in three different parts of one leaf including petioles and midribs, leaf blade lower and upper parts. In their research, some fungal taxa that were not reported based on their morphological or cultural characteristics were identified using PCR-DGGE (Duong et al., 2006).

Metagenomics and metabarcoding are sequence-based and gene function analyses of collective microbial genomes or microbial amplicons contained in an environmental sample. In a complex ecosystem such as the soil, plant roots and stems, the identity and function of microbes can be revealed by metabarcoding analysis of taxonomic genes and/or the functional genes of microbes, respectively (Sengupta et al., 2017; Simon & Daniel, 2011; Tian et al., 2015). With the advent of next generation sequencing (NGS), sequence-based studies have been significantly accelerated and applied to a broader range of microbial communities. Next generation sequencing offers the advantages of being less time-consuming and substantially less costly than Sanger sequencing and also provides a great depth of sequencing (Mizrahi-Man et al., 2013). Illumina sequencing is a commonly-used NGS platform. It generates shorter sequences than Sanger sequencing and can process millions of sequence reads in parallel as compared to Sanger that processes 96 at a time (Mardis, 2008).

Metabarcoding such as amplicon sequencing based on NGS is an effective way to analyse the hypervariable regions of the taxonomic genes of bacteria and fungi. For example, Bodenhausen et al. (2013) effectively evaluated the population structure of bacterial communities associated with the leaves and roots of wild *Arabidopsis thaliana* using 454 pyrosequencing based on the amplified V5, V6 and V7 of the 16S rRNA gene. They found that leaf and root endophytic communities showed no difference in the taxa richness, but differed in community composition

(Bodenhausen et al., 2013). Bazzicalupo et al. (2013) successfully evaluated the diversity of leaf-associated fungal communities by analysing ITS1 and ITS2 rRNA regions with 454 pyrosequencing. In their research, the combined-use of ITS1 and ITS2 provided a comprehensive characterisation of hyperdiverse fungal communities due to the taxon-specific differences in the taxonomic information they carried.

Shotgun metagenomics (screening or sequencing of libraries of randomly isolated DNA fragments) allows the full microbial genomes in a sample to be sequenced (Zarraonaindia et al., 2013). Uroz et al. (2013) investigated the microbial structure in the complex ecosystem of mineral horizon and organic horizon of acidic forest soils by generating 14 Gbp sequences with the combined use of pyrosequencing and Illumina sequencing platforms. Based on the analysis of sequences, they found that the number of bacteria, Chordata, Arthropoda and Ascomycota were increased in the organic soil horizon, while Archaea was enriched in the mineral soil horizon (Uroz et al., 2013). From the metagenomic sequencing data, the presence of gene sequences coding for plant cell-wall degrading enzymes which determines the colonisation by endophytes or as a pathogenic factor were detected (Tian et al., 2015).

However, there are also limitations with the use of molecular methods. For example, endophyte diversity could be affected by the DNA extraction procedure used (Maropola et al., 2015). In addition, PCR bias causes underestimation of microbial richness because certain taxonomic groups are not detected by the PCR primers used (Hong et al., 2009). For example, excessively long ITS barcodes and introns within barcodes may prohibit amplification and sequencing of some taxa (Lindahl et al., 2013). Further, PCR technique amplifies DNA from both viable and dead microbes, resulting in an overrepresentation of some microbial taxa.

1.6.4.3 Evaluation of microbial communities using DGGE and metabarcoding

DGGE analysis provides a community fingerprint which can directly reveal differences in the microbial composition of different environmental samples. Representatives can then be taxonomically identified by sequencing bands of interest (Chng et al., 2015). Metabarcoding, using NGS, is a newer technique that also produces sequence information from complex microbial communities. However, in comparison with metabarcoding, DGGE provides less information and a lower level of resolution. For example, DGGE fragments with similar sequences or with the same melting behaviour may not be resolved in the DGGE gel (Ercolini, 2004). In contrast, for metabarcoding millions of sequences are obtained simultaneously from a sample. Samples are distinguished by barcodes incorporated into the primers used in metabarcoding studies (Bazzicalupo et al., 2013; Lazarevic et al., 2009; Uroz et al., 2013). However, metabacoding is

more expensive than DGGE especially when a large number of samples must be processed. In this project, a large number of trees in multiple orchards were assessed for multiple microbial taxa (fungi and bacteria) and many of these at two time points (spring and autumn). Analysing all samples using DGGE is a more cost effective approach that will give sufficient insight into the microbial communities in the samples. Another potential disadvantage of DGGE is that it does not enable abundance of microbiomes in the community composition to be known although some interpretation can be made from band intensity. In contrast, the number of reads generated per operational taxonomic unit (OTU) in metabarcoding can be used to provide a relative abundance of microbiomes within the sample. Therefore, a combination of approaches allows a rapid and cost effective way to reveal endophyte community composition.

1.7 Apple endophytes

Members of the endophyte communities of various apple tissues have been identified in previous studies using either culture dependent or independent methods. For example, using terminal restriction fragment length polymorphism, Hirakue and Sugiyama (2018) investigated the richness, composition, and abundance of the foliar endophyte communities in three apple cultivars ('Kogyoku', 'Fuji' and 'Orin') in Japan. By cloning terminal restriction fragments (T-RFs), 19 endophytic fungal species including *Cladosporium tenuissimum*, *Lophiostoma* sp. and *Leptosphaerulina chartarum*, and seven endophytic bacterial species including *Pantoea agglomerans*, *Pseudomonas* sp. and member of the Burkholderiales were identified (Hirakue & Sugiyama, 2018). Using culture dependant methods, filamentous fungi including *Colletotrichum*, *Xylaria*, *Botryosphaeria*, *Alternaria*, *Cladosporium*, *Epicoccum* and *Fusarium* were identified by Camatti-Sartori et al. (2005), as member of the endophyte community of a wider range of apple tissues (leaves, flowers and fruit). High-throughput amplicon sequencing of the total DNA from apple stem tissue identified the dominant bacterial (Proteobacteria, Fimicutes) and fungal phyla (Ascomycota, Zygomycota and Basidiomycota) in different apple varieties and rootstocks combinations (Liu et al., 2018). For apple roots, *Bacillus*, *Pseudomonas* and *Burkholderia* were identified using a combination of culturing methods and cloning analysis by Bulgari et al. (2012). Similar results were reported by Muresan (2017) in that *Bacillus* species was the predominant endophytic bacterial genus (half of the recovered bacterial isolates) isolated from apple roots, or leaves/petioles. They also reported that *Penicillium* or *Trichoderma* species were the predominant fungal species isolated from apple roots, accounting for 60% of the recovered fungal isolates.

Apple endophytes have been studied for their plant growth promotion and biocontrol potential. Endophytic bacteria isolated from apple tree buds by Miliūtė and Buzaitė (2011) were found to have plant growth promoting properties such as producing the plant hormone IAA and siderophores, solubilising calcium phosphate, fixing nitrogen, and antibacterial activity against *Salmonella typhimurium*. Arbuscular mycorrhizal fungal (AMF) colonisation of apple roots was shown to be affected by cultivar and artificial inoculation with AMF increased resistance to *N. ditissima* (Berdeni et al., 2018). It indicated disease resistance of cultivars maybe closely related to the endophyte community in different apple cultivars. For example, Hirakue and Sugiyama (2018) found that the resistance of apple cultivars to Alternaria leaf spot (*Alternaria mali*) was related to the fungal endophyte diversity in leaves. Moreover, Liu et al. (2018) reported that apple endophyte community determined by Illumina-based next generation sequencing was associated to cultivar and cultivar-rootstock combinations, and cultivars with closer pedigree had more similar microbial communities than those with distantly-related pedigrees. In their study, they also found a greater number of growth-promoting bacteria taxa were in the more vigorous rootstocks M.M.111 than in the dwarfing rootstock M.9.

In addition, the apple endophyte community has been shown to be affected by other factors such as age of apple leaves (Afandhi et al., 2017), infection level of apple proliferation disease (*Candidatus* Phytoplasma mali) (Bulgari et al., 2012), cultivation systems (conventional, integrated and organic) (Camatti-Sartori et al., 2005) and soil conditions (replant, fallow and sterilised soil) (Manici et al., 2013). These results suggest that the composition of the apple endophyte community are correlated with disease occurrence and plant growth rate. Therefore, identification of apple endophytes with biocontrol and plant growth benefits could be a strategy to enhance apple growth and resilience to pathogen challenge.

The effect of other factors such as tissue type, site, region and season on apple endophyte community is unknown, though they have been found to influence endophyte communities in other plant species (Arnold et al., 2007; Knief et al., 2010; Peršoh, 2013; Rather et al., 2018).

1.8 Aims and objectives of this research

There is limited research on the endophytic communities of apple and their bioactivity in relation to their ability to suppress pathogens. The overarching hypothesis of this project is that 'members of the apple endophyte community are able to inhibit *N. ditissima* infection of apples'. This project aims to identify endophytes of apples that are able to inhibit infection by *N. ditissima* and demonstrate that these are able to reduce infection by the pathogen *in planta*. To achieve this aim, four objectives were developed as outlined:

Objective 1: To identify factors affecting endophyte communities in apple shoots (Chapter 2).

Objective 2: To identify the *in vitro* biocontrol activity of culturable endophytes against *N. ditissima* (Chapter 3).

Objective 3: To determine the colonisation and persistence of selected antagonistic endophytic bacteria in detached apple shoots (Chapter 4).

Objective 4: To evaluate the biocontrol activity of selected antagonistic bacterial endophytes against *N. ditissima* in detached and attached apple shoots (Chapter 5).

Chapter 2 Identification of factors affecting endophyte community of apple shoots

2.1 Introduction

DNA-based techniques are improving our knowledge of the complete microbiome of plants (culturable and unculturable ones), thus expanding our understanding on endophyte community structure (Manter et al., 2010; Peršoh, 2013). Endophyte communities have been shown to be diverse in plants and to play an important role in the ecology of the host plants. A study by Kang et al. (2016) using DGGE showed that the endophytic bacterial community differed in pepper plants (*Capsicum annuum* L. cv. Nokkwang) when cultivated in different soils collected from different geographic locations in Korea, with the proportion of antagonistic bacterial isolates identified also varying in plant samples from the different soils. Also, using DGGE, Patel and Archana (2017) demonstrated diazotrophic endophyte bacterial communities were similar in different plant tissues (leaf, stem and root) of cereal plants (*Poaceae*), which revealed their diverse but non-specific colonisation in cereal plants and the beneficial traits in enhancing plant growth.

Endophyte communities are influenced by several factors, such as host species and tissue types (Peršoh, 2013), cultivars with different resistance to plant pathogens (Hirakue & Sugiyama, 2018; Sessitsch et al., 2002), leaf age (Afandhi et al., 2017), disease infection level (Bulgari et al., 2012), geographical scales (Arnold et al., 2007), management practice (Camatti-Sartori et al., 2005) and season (Rather et al., 2018). In this study, factors affecting the endophyte community composition of apple focused on tissue type, cultivar, region, site and season. Endophytic microbial communities including total bacteria, total fungi, α, β, γ-proteobacteria and actinobacteria were evaluated for their community similarity and richness as affected by tissue type, cultivar and site using environmental PCR and DGGE. The subsequent analyses then focused on total bacteria, total fungi, γ-proteobacteria and actinobacteria to evaluate the effect of region and interaction of season, site and cultivar. The cultivars in this study were selected based on their reported susceptibility to European canker with 'Royal Gala' reported to be less susceptible than 'Scifresh' (Bus et al., 2015) and the susceptibility of 'Braeburn' being unknown. Management practice (IFP-managed versus organic) and infection level were not evaluated here, because sample size was not sufficient.

The objectives of this study were (i) to determine endophyte profiles affected by orchard factors in New Zealand, and (ii) to determine the correlation between endophyte colonisation and varietal resistance with composition of the endophyte community.

2.2 Materials and methods

2.2.1 Sampling strategies

2.2.1.1 Main sampling in spring (M1) and autumn (M2) for commercial varieties

Sampling was conducted in spring when leaves were fully-opened (November-December 2015, M1 sampling) and autumn close to harvest and before leaf fall (April 2016, M2 sampling) for commercial varieties including 'Royal Gala', 'Braeburn', 'Scifresh' and 'Scilate' from commercial orchards in Nelson (spring and autumn) and Hawke's Bay (spring only) (Figure 2.1). One-year old shoots on the second side branch from the lowest main branch were sampled from mature (over 5-years old) and healthy trees (with standard growth rate, no nutrient deficiency and no obvious foliar or trunk disease symptoms and insect damage). Apple tissue samples from 12 blocks (five sites) (Appendix A2.1) were used for analysis of the complete microbial endophyte community composition affected by orchard factors including tissue type, region, site, cultivar and season.

Six trees were randomly selected for sampling from three rows of each block with low European canker infection (European canker infection rate lower than 30% in 2015, defined by this study). In each block, the first and last rows of the block and the first three and the last three trees in a row were not selected. The row for starting sampling was randomly selected using a dice. Then, the adjacent two rows were selected for sampling. Two trees were sampled from each row, giving a total of six trees from each block. In each row, the first healthy tree for sampling was randomly selected using a dice, with the next but one healthy tree within the row also sampled.

The collected shoots included the second and third fully-opened leaves counted from the tip, and green and woody stem portions. The young stem portion from the second fully extended leaf downwards (referred to as green stem) and the woody stem portion from the base of the shoot upwards (referred to as woody stem) of at least 7 cm long for each stem portion was collected to enable the subsequent sampling to be carried out (Figure 2.1). The shoots were placed in paper bags and transported in a chilly bin to the laboratory at Lincoln University for processing.

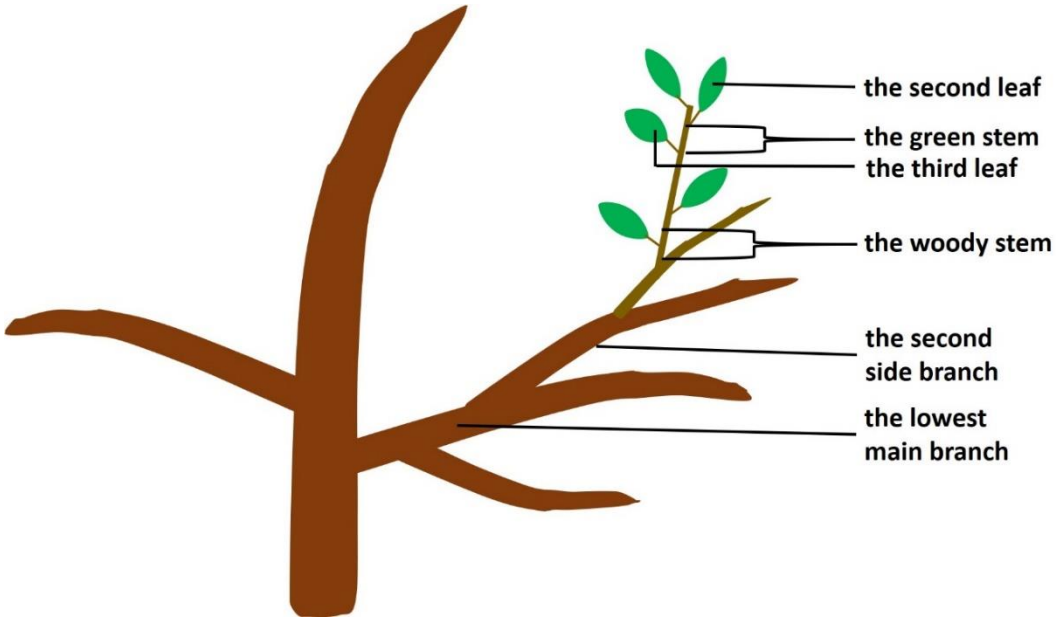

Figure 2.1 Diagram illustrating the 1-year old shoot including two leaves and two stem portions sampled from the second side branch.

2.2.2 Surface sterilisation of plant tissues

The 2nd and 3rd fully-opened leaves, green stem and woody stem portions from each shoot were surface sterilised based on the procedure commonly used and shown to be effective for isolating endophytes from plant material (Purushotham Balraj, 2017; Wicaksono, 2016). Tissue samples were washed using tap water, air dried in a laminar flow hood, and surface sterilised. The tissue samples were soaked in 96% ethanol for 10 s, 2.5% sodium hypochlorite solution for 3 min for stem samples and 2 min for leaf samples, and then washed three times in sterile water for 1 min to remove the sterilising agents. After surface sterilisation, all the leaf and stem samples were cut into smaller segments (approx. 0.5 cm long) and stored at -80°C before DNA extraction. The remaining samples were used for isolation of culturable endophytes as described in Chapter 3. The water of final rinse was plated on R2A (Difco, Becton, Dickinson and Company) to verify the effectiveness of the surface sterilisation protocol.

2.2.3 DNA extraction

For each of the surface sterilised plant tissue samples, three leaf pieces (0.5 mm wide) for each leaf and three stem segments with bark (0.5 mm thickness) for each stem segment were soaked in 300 - 400 µL sterile millipore water in light transparent 0.7 mL tubes (Axygen, USA) and 1 µL 20 mM propidium monoazide (PMA) (Biotium, USA) was added to each tube as described by Wicaksono (2016). PMA was used to prevent amplification of DNA from the surface, non-endophyte, microbes which have been killed by the surface sterilisation procedure (Carini et al.,

2017; Nocker et al., 2007). The samples were incubated in the dark for 5 min, followed by exposure to light using a 650-W halogen light for 7 min to cross-link the PMA with DNA from non-viable microbial cells. After PMA treatment, tissue samples were ground with liquid nitrogen using sterile mortars and pestles. DNA was extracted from the ground plant samples using PowerPlant™ DNA isolation kit (MoBio Laboratories, Carlsbad, USA). DNA quality and concentration were measured by spectrophotometry (nanodrop) and gel electrophoresis (1%).

2.2.4 Polymerase chain reaction (PCR) amplification

All the PCR reactions were performed in a total reaction mixture of 25 µL consisting of 18.75 µL ultra-pure water (Life Technologies, Thermo Fisher Scientific, USA), 2.5 µL 10 × PCR buffer with 20 mM $MgCl_2$ (a final concentration of 2 mM $MgCl_2$), 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.2 µM of each forward and reverse primers, 1.25 U FastStart *Taq* DNA Polymerase (Roche, Roche Custom Biotech, Switzerland) and 1 µL DNA template. For the nested PCRs, the primary PCR was conducted as described. Then, 1 µL of the primary PCR product was used as DNA template for the secondary PCR. The primers and thermal cycles used for the total bacteria, total fungi, proteobacteria (α, β and γ) and actinobacteria are shown in Table 2.1.

PCR products were checked for amplification of endophyte taxa with electrophoresis in 1% agarose gels (in 1 × TAE, Tris-Acetate-EDTA) at 100 V for 45 min and then stained in ethidium bromide staining solution (0.5 µg/mL, Biorad, USA) for 20 min, and destained by soaking in tap water for 10 min. The gels were visualised in a UV transilluminator (UNITEC Cambridge, Total Lab Systems, New Zealand). For the nested PCRs, only the secondary PCR products were checked with the electrophoresis.

Table 2.1 Group-specific 16S ribosomal RNA gene and 18S ribosomal RNA gene primers and thermal cycles applied in the nested PCRs for different microbial groups.

Microbial group	Primer	Sequence (5'-3')	PCR conditions	Reference
Total bacteria	357F GC	**39 bp GC clamp**-CCT ACG GGA GGC AGC AG	95°C for 3 min 35 cycles of 94°C for 1 min,	Muyzer et al. (1993)
	518R	ATT ACC GCG GCT GCT GG	55°C for 1 min, 72°C for 1 min 72°C for 7 min	
Total fungi (primary PCR)	AU2	TTT CGA TGG TAG GAT AG**D** GG (where **D**: A or G or T)	95°C for 3 min 35 cycles of 94°C for 1 min,	Vandenkoornhuyse et al. (2002)
	AU4	**R**TC TCA CTA AGC CAT TC (where **R**: A or G)	50°C for 1 min, 72°C for 1 min 72°C for 7 min	
Total fungi (secondary PCR)	FF390	CGA TAA CGA ACG AGA CCT	95°C for 2 min 8 cycles of 95°C for 30 s, 55°C	Vainio and Hantula (2000)
	FR1-GC	**40 bp GC clamp** [(1)]-A**I**C CAT TCA ATC GGT A**I**T (where **I**: inosine)	for 30 s, 72°C for 1 min; 27 cycles of 95°C for 30 s, 47°C for 30 s, 72°C for 1 min 72°C for 7.5 min	
α-proteobacteria (primary PCR)	F203A	CCG CAT ACG CCC TAC GGG GGA AAG ATT TAT	94°C for 5 min 30 cycles of 94°C for 1 min,	Nübel et al. (1996), Gomes et al. (2001), da Silva et al. (2013)
	L1401	GCG TGT GTA CAA GAC CC	56°C for 1 min, 72°C for 2 min 72°C for 10 min	
α-proteobacteria (secondary PCR)	341F GC	**40 bp GC clamp** [(1)]-CCT ACG GGA GGC AGC AG	96°C for 4 min 30 cycles of 96°C for 1 min,	Muyzer et al. (1993), Mühling et al. (2008)
	518R	ATT ACC GCG GCT GCT GG	56°C for 1 min, 74°C for 1 min 74°C for 10 min	
β-proteobacteria (primary PCR)	Beta359F	GGG GAA TTT TGG ACA ATG GG	96°C for 4 min 30 cycles of 96°C for 1 min,	Ashelford et al. (2002), Mühling et al. (2008)
	Beta682R	ACG CAT TTC ACT GCT ACA CG	63°C for 1 min, 74°C for 1 min 74°C for 10 min	

Table 2.1 Continued

Microbial group	Primer	Sequence (5'-3')	PCR conditions	Reference
β-proteobacteria (secondary PCR)	518F GC	**40 bp GC clamp** [(1)]-CCA GCA GCC GCG GTA AT	96°C for 4 min 30 cycles of 96°C for 1 min,	Ashelford et al. (2002), Muyzer et al. (1993), Mühling et al. (2008)
	Beta682R	ACG CAT TTC ACT GCT ACA CG	60°C for 1 min, 74°C for 1 min 74°C for 10 min	
γ-proteobacteria (primary PCR)	Gamma395F	CMA TGC CGC GTG TGT GAA (where **M**: A or C)	96°C for 4 min 30 cycles of 96°C for 1 min,	Mühling et al. (2008)
	Gamma871R	ACT CCC CAG GCG GTC **D**AC TTA (where **D**: A or G or T)	54°C for 1 min, 74°C for 1 min 74°C for 10 min	
γ-proteobacteria (secondary PCR)	518F GC	**40 bp GC clamp** [(1)]-CCA GCA GCC GCG GTA AT	96°C for 4 min 30 cycles of 96°C for 1 min,	Muyzer et al. (1993), Lee et al. (1993), Mühling et al. (2008)
	Gamma785R	CTA CCA GGG TAT CTA ATC C	56°C for 1 min, 74°C for 1 min 74°C for 10 min	
Actinobacteria (primary PCR)	F243	GGA TGA GCC CGC GGC CTA	95°C for 5 min 35 cycles of 94°C for 1 min,	Purushotham et al. (2018)
	R1494	TAC GGC TAC CTT GTT ACG AC	60°C for 1 min, 72°C for 2 min 72°C for 10 min	
Actinobacteria (secondary PCR)	F341GC	**40 bp GC clamp** [(2)]-CCT ACG GGA GGC AGC AG	95°C for 3 min 35 cycles of 94°C for 30 s, 53°C	Nimnoi et al. (2011)
	R534	ATT ACC GCG GCT GG	for 1 min, 72°C for 1 min 72°C for 5 min	

39 bp GC clamp: 5'-CGC CCG CCG CGC GCG GCG GGC GGG CGG GGG CAC GGG GGG-3' (Muyzer et al., 1993).

40 bp GC clamp [(1)]: 5'-CCC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GCC G-3' (Vainio & Hantula, 2000).

40 bp GC clamp [(2)]: 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3' (Nimnoi et al., 2011).

2.2.5 Denaturing gradient gel electrophoresis (DGGE)

DGGE were performed with Cipher DGGE Electrophoresis System (C.B.S. Scientific, USA) based on the method reported by Wicaksono et al. (2016). Five to eight µL PCR product with 3 µL 2 × loading dye (Appendix A2.2.1) were loaded onto an 8% (w/v) polyacrylamide gel (acrylamide/bis solution, 37.5:1; Appendix A2.2.2) with 45%-65% linear denaturing gradient for total bacteria, 25%-55% for total fungi, 40%-60% for α-proteobacteria, 40%-55% for β-proteobacteria and 35%-50% for actinobacteria. For γ-proteobacteria, 7% polyacrylamide gels (Appendix A2.2.2) were used with 40%-60% linear denaturing gradient. The gels were run in 0.5 × TAE buffer at 58°C at 90 V for 16 h for total bacteria and total fungi, at 60 V for 18 h for α-proteobacteria and β-proteobacteria, at 85 V for 16 h for γ-proteobacteria and at 130 V for 6 h for actinobacteria. After electrophoresis, gels were washed with reverse osmosis (RO) water to remove the remaining TAE buffer, and then fixed by shaking in 250 mL 1 × fixation solution (Appendix A2.2.5) for 10 min. The gels were the stained in silver staining solution (Appendix A2.2.6) for 10 min. The silver stain was then decanted and the gels washed with RO water and shaken for 2 min in 200 mL RO water. The gels were then developed by shaking in developer solution (Appendix A2.2.7) for 40 min. Each gel was then shaken again in 250 mL 1 × fixation solution for 5 min before being photographed, and the photographs used for analysis of the microbial communities. Finally each gel was shaken in 200 mL RO water for 2 min and then preserved in 200 mL Cairn's preservation solution (Appendix A2.2.8) for 7 min. The preserved gel was covered with a piece of cellophane and dried at 60°C for 6 h.

2.2.6 Experiment design and data analysis

2.2.6.1 Experiment design

Initially the effects of tissue type, site and cultivar on the endophyte microbial community of apple shoots were determined using DGGE for all the six microbial groups (Table 2.1). Samples were collected and analysed in four tissue types of 'Royal Gala' from three sites (sites 2, 4 and 6) and four tissue types of 'Braeburn' from site 2 in spring (Appendix A2.1). There were six replicate shoots per cultivar per site, resulting in six replicates of each tissue type per cultivar per site.

Further, the effects of region, season and a further cultivar analysis with 'Scifresh' included on the total bacteria, total fungi, γ-proteobacteria and actinobacteria endophyte community in woody stems were evaluated. For region assessment, samples were collected and analysed from each of two sites in Nelson (sites 4 and 1) and Hawke's Bay (sites 6 and 5) in spring only (Appendix A2.1). The factor of site was nested in the factor of region. For season assessment, samples were collected and analysed in two seasons, spring and autumn, at each of three sites (sites 1, 2 and 4) for 'Royal Gala' and 'Braeburn'. For the further cultivar assessment, samples were collected and analysed for

'Royal Gala', 'Braeburn' and 'Scifresh' at two sites (sites 2 and 6) in spring only, in order to assess cultivars with variable resistance to European canker. Three replicate shoots per cultivar per site were used, resulting in three replicates for each tissue type per cultivar per site.

2.2.6.2 Data analysis

Analysis of DGGE gel pictures for microbial communities were performed in Phoretix 1D (Totallab, UK), resulting in the generation of a binary matrix indicating the presence (1) or absence (0) of each band detected in all the lanes. The effect of the different factors on the endophytic fungal/bacterial community similarity and richness were analysed. One band was considered as one bacterial /fungal taxa. Resemblance matrices for community profiles were built by calculating similarities between each pair of samples based on Jaccard similarity index (S7) (Clarke & Warwick, 2001) in Primer 7 (Primer-E, Plymouth Marine Laboratory, UK). Main and pairwise PERMANOVA tests were used to test the statistical difference in the endophyte communities affected by the factor(s) and interaction effect of factors. Main and pairwise PERMANOVA tests were used to test the statistical difference between endophytic bacterial/fungal communities among treatment factors, with both permutation and Monte Carlo P values used to determine the significant levels. The Monte Carlo P value was used when the number of unique permutations was low (Anderson et al., 2008). Nonmetric multidimensional scaling (nMDS) plots generated in Primer 7 were used to visualise the similarity in the endophyte community composition affected by the treatment factors and interactions. The number of bands per lane was used as a diversity indicator of the bacterial/fungal taxa richness. The effect of treatment factors on endophytic bacteria/fungal richness was analysed using a general linear model (GLM) (data fit a normal distribution) in SPSS Statistics 24 (IBM, USA). When the effect of a factor was significant by GLM, a pairwise test was then conducted based on least significant difference test (LSD). All null-hypothesis significance tests were performed with $\alpha = 0.05$. For the pairwise tests for endophyte community similarity/richness, if there was interaction of factors, pairwise tests of a factor were conducted within each level of the other factor(s), respectively. If there was no interaction of factors, pairwise tests of a factor was conducted between the levels of this factor itself because it did not interact with the other factor(s).

2.3 Results

2.3.1 Effect of tissue type and the interaction with cultivar/site on endophyte communities

In this section, the endophyte communities in four tissue types in two cultivars and, for one cultivar across sites was examined. The factor of cultivar could only be assessed for site 2 as this was the only site in which four tissues of both 'Royal Gala' and 'Braeburn' were collected. In all other sites

only stems were collected for 'Braeburn'. The factor of site could only be examined for 'Royal Gala' as this was the only cultivar for which all four tissues were collected across three sites.

2.3.1.1 Total bacteria

Tissue type and cultivar

The interaction of tissue type and cultivar significantly affected the total bacterial community (Table 2.2). Total bacterial communities from each cultivar clustered together, with those in 'Braeburn' more homogenous and also clustered by tissue type when compared to 'Royal Gala' (Figure 2.2, Appendix A2.3). Pairwise tests on cultivar showed that the effect of cultivar on the bacterial community was significant for all tissue types (Appendix A2.4). Pairwise tests for each cultivar showed that 2nd and 3rd leaves were not different from each other irrespective of cultivar (Appendix A2.4). For 'Braeburn', leaves and stems differed significantly, with green stems also differing from woody stems. In contrast, for 'Royal Gala', only woody stems were different from all other tissues. For 'Royal Gala' green stems and 2nd leaves were similar, but not to 3rd leaves.

The interaction of tissue type and cultivar significantly affected total bacterial richness (Table 2.2). Pairwise tests on cultivar showed that the effect of cultivar on the richness of the bacterial community was significant for leaves but not stems, with 'Braeburn' communities typically more rich than 'Royal Gala' (Appendix A2.4). Pair wise tests on the four tissue types showed that, for 'Royal Gala', total bacterial community richness differed between the 2nd leaf and all other tissues, with the 2nd leaf being the least rich. For 'Braeburn' the 3rd leaf was also richer than the 2nd leaf. For 'Braeburn' woody stem was less rich than 3rd leaf and green stem (Appendix A2.4).

Table 2.2 Effect of tissue type and cultivar on the similarity and richness of total bacterial community identified using DGGE.

	Total bacteria	
Factors	Similarity[#]	Richness[$]
Tissue type	0.001**	0.004**
Cultivar	0.001**	< 0.001**
Tissue type × cultivar	0.001**	0.028*

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

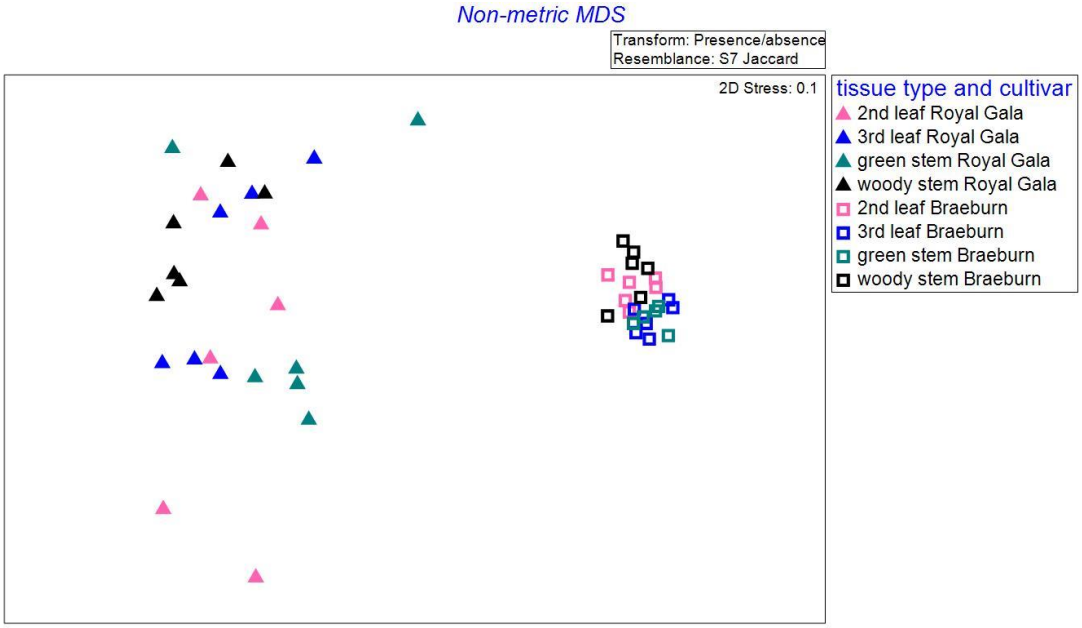

Figure 2.2 Nonmetric multidimensional scaling (nMDS) plot showing total bacterial community in two cultivars 'Braeburn' (□), 'Royal Gala' (▲); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

Effect of tissue type and site on 'Royal Gala'

The interaction of tissue type and site significantly affected the total bacterial community (Table 2.3). Total bacterial communities from sites 4 and 6 were similar and separated from that in site 2, with no cluster by tissue type (Figure 2.3, Appendix A2.5). Pairwise comparisons on site showed that irrespective of tissue type site 2 always differed from the other two sites. For site 4 only the bacterial communities in the 3rd leaf and green stem differed from site 6. Pairwise comparisons of tissue types showed that the woody stems in site 2 differed to all other tissues collected at that site. For this site, green stems also differed from the 3rd leaf. In site 4 green stems and 2nd leaf were the only tissues that differed, whereas, in site 6 there were significant differences between leaf groups and between woody stems and 3rd leaf.

Site significantly affected the richness of total bacterial community, but not tissue type or the interaction of tissue type and site (Table 2.3). Site 2 was less rich than sites 4 and 6 but sites 4 and 6 were not different from each other (Appendix A2.6).

Table 2.3 Effect of tissue type and site on the similarity and richness of total bacterial community identified using DGGE.

	Total bacteria	
Factors	Similarity[#]	Richness[$]
Tissue type	0.001**	0.089
Site	0.001**	< 0.001**
Tissue type × site	0.002**	0.101

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

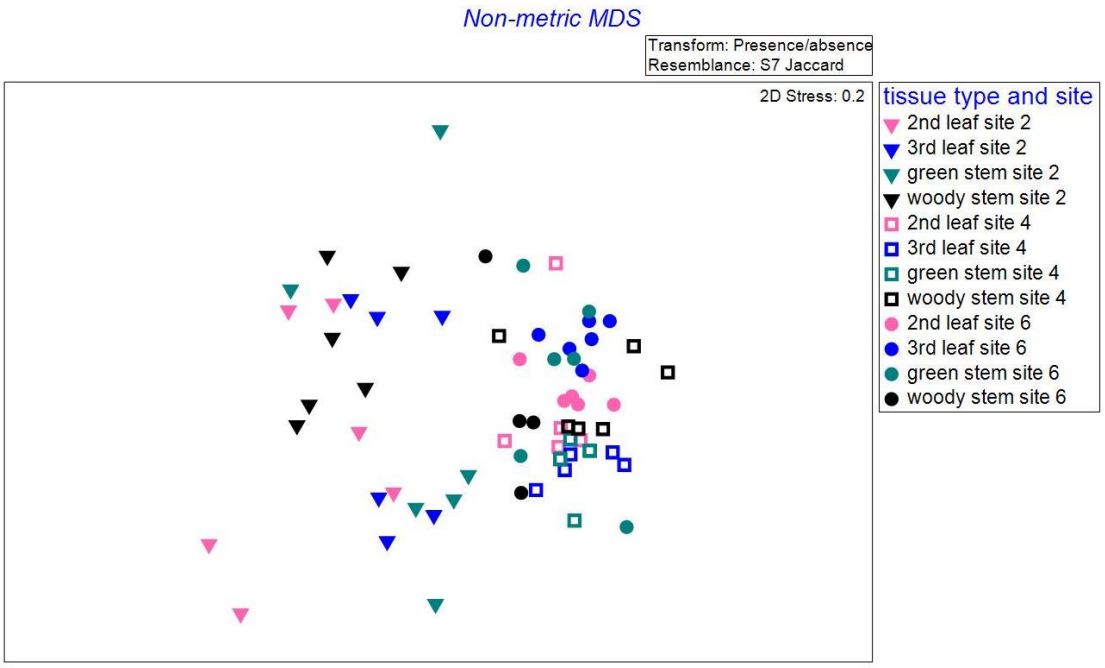

Figure 2.3 Nonmetric multidimensional scaling (nMDS) plot showing total bacterial community from three sites; site 2 (▼), site 4 (□) and site 6 (●); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

2.3.1.2 Total fungi

Tissue type and cultivar

The interaction of tissue type and cultivar significantly affected the total fungal community (Table 2.4). The total fungal community in 'Braeburn' was more homogenous than that in 'Royal Gala' but did not cluster separately from 'Royal Gala', with no cluster by tissue type (Figure 2.4, Appendix A2.7). Pairwise tests on cultivar showed that the effect of cultivar was significant for all tissue types except green stem (Appendix A2.8). In both 'Royal Gala' and 'Braeburn', total fungal community was similar between leaf groups, and between stem groups (Appendix A2.8). For 'Royal Gala', the

total fungal community only differed between woody stem and the 2nd leaf. For 'Braeburn', the significant difference was only between woody stem and 3rd leaf.

Total fungal richness was not affected by tissue type, cultivar or interaction between them (Table 2.4).

Table 2.4 Effect of tissue type and cultivar on the similarity and richness of total fungal community identified using DGGE.

	Total fungi	
Tissue type and cultivar	Similarity#	Richness$
Tissue type	0.018*	0.216
Cultivar	0.001**	0.333
Tissue type × cultivar	0.020*	0.302

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

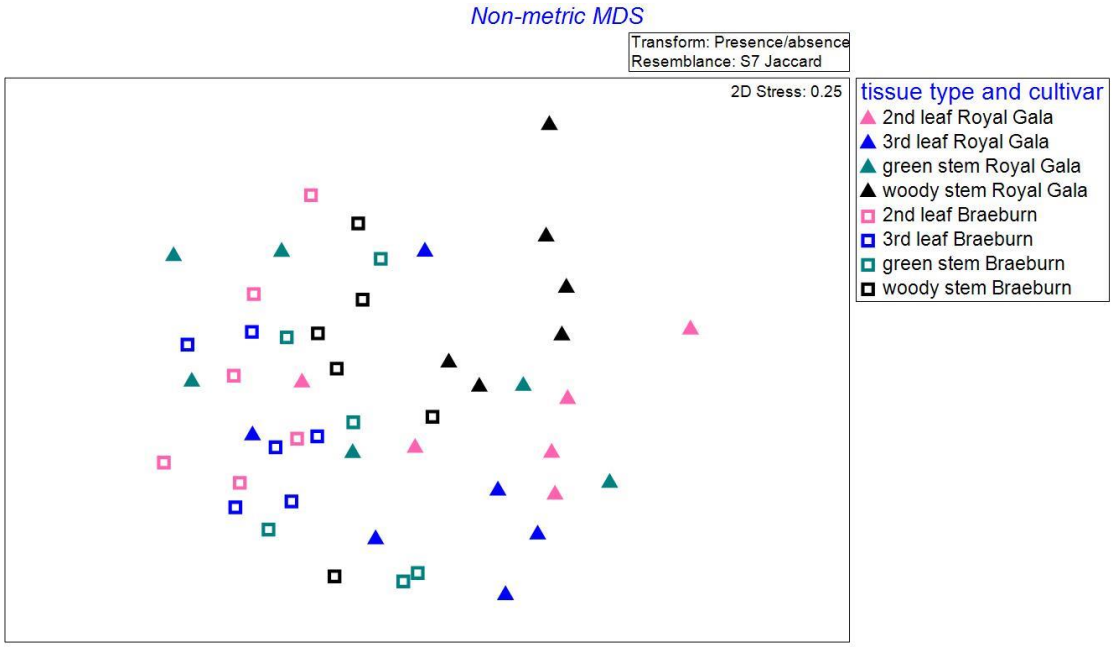

Figure 2.4 Nonmetric multidimensional scaling (nMDS) plot showing total fungal community in two cultivars 'Braeburn' (□), 'Royal Gala' (▲); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

Effect of tissue type and site on '*Royal Gala*'

The interaction of tissue type and site significantly affected the total fungal community (Table 2.5). The total fungal community did not cluster by tissue type or site. Total fungal community in site 4 and site 6 were more similar to each other than to site 2 (Figure 2.5, Appendix A2.9). Pairwise tests on tissue type showed that in site 4 and site 6 the total fungal community in the 2nd leaf was significantly different from the other three tissue types (Appendix A2.10). Also, for these two sites total fungal community in the 3rd leaf differed from that in woody stem. In site 2, total fungal community was only different between woody stem and the 2nd leaf. Pairwise tests on site showed that the effect of site on the total fungal community was significant for all tissue types except the 3rd leaf between site 4 and site 6 (Appendix A2.10).

Total fungal richness was significantly affected by the interaction of tissue type and site (Table 2.5). Pairwise tests showed no difference in total fungal community richness between tissue types in site 2 (Appendix A2.10). In contrast, for site 4 the total fungal community was richer in the 2nd leaf than the other three tissues. For site 6 total fungal community richness was similar between woody stem and the 2nd leaf, but they were both significantly higher than the 3rd leaf, with the least richness in green stem. Pairwise tests on site showed that total fungal richness was not affected by site for the 3rd leaf and green stems. In contrast, for the 2nd leaf and woody stem total fungal richness in site 6 was significantly higher than that in site 2. In woody stem, total fungal community in site 6 was richer than in site 4, with no significant difference between site 2 and site 4 (Appendix A2.10).

Table 2.5 Effect of tissue type and site on the similarity and richness of total fungal community identified using DGGE.

	Total fungi	
Tissue type and site	Similarity[#]	Richness[$]
Tissue type	0.017*	0.001**
Site	0.001**	0.002**
Tissue type × site	0.001**	0.004**

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

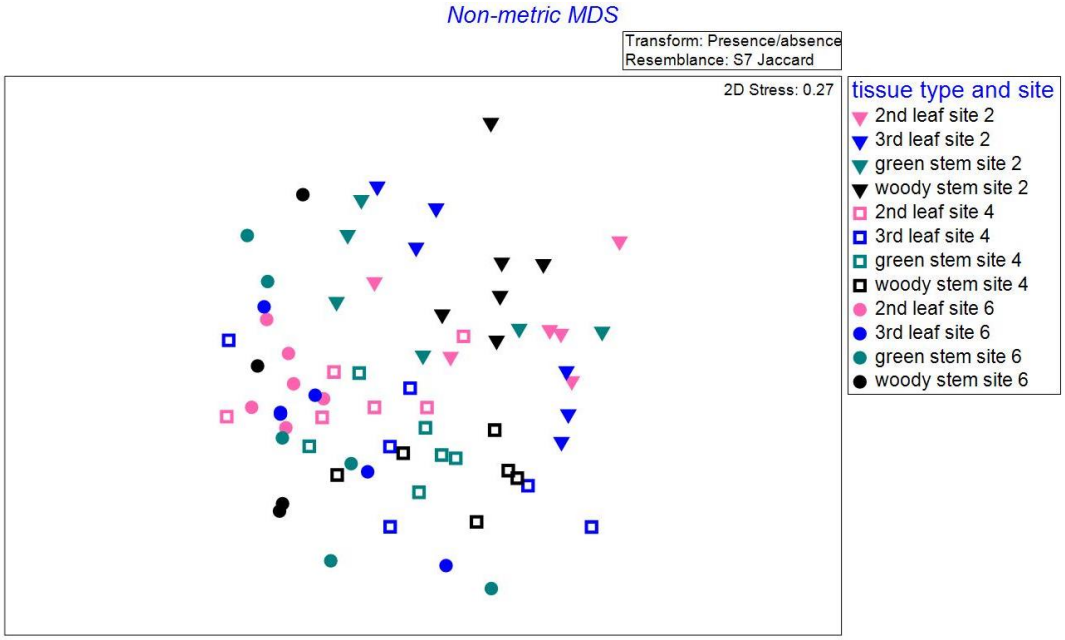

Figure 2.5 Nonmetric multidimensional scaling (nMDS) plot showing total fungal community from three sites; site 2 (▼), site 4 (□) and site 6 (●); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

2.3.1.3 α-proteobacteria

Tissue type and cultivar

The interaction of tissue type and cultivar significantly affected the α-proteobacterial community (Table 2.6). The α-proteobacterial community in each cultivar clustered together, with 'Braeburn' more homogenous than 'Royal Gala' (Figure 2.6, Appendix A2.11).

For 'Braeburn', the α-proteobacterial community in the 2nd leaf, 3rd leaf and green stem were more similar to each other, than the woody stem. Pairwise tests showed that tissue type did not affect α-proteobacterial communities in 'Royal Gala', while for 'Braeburn' α-proteobacterial community was significantly different between all tissue types (Appendix A2.12). Pairwise tests showed that cultivar had a significant influence on α-proteobacterial communities in all tissue types.

α-proteobacterial richness was not affected by tissue type, cultivar or their interaction (Table 2.6).

Table 2.6 Effect of tissue type and cultivar on the similarity and richness of α-proteobacterial community identified using DGGE.

	α-proteobacteria	
Tissue type and cultivar	Similarity[#]	Richness[$]
Tissue type	0.001**	0.725
Cultivar	0.001**	1.000
Tissue type × cultivar	0.002**	0.228

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

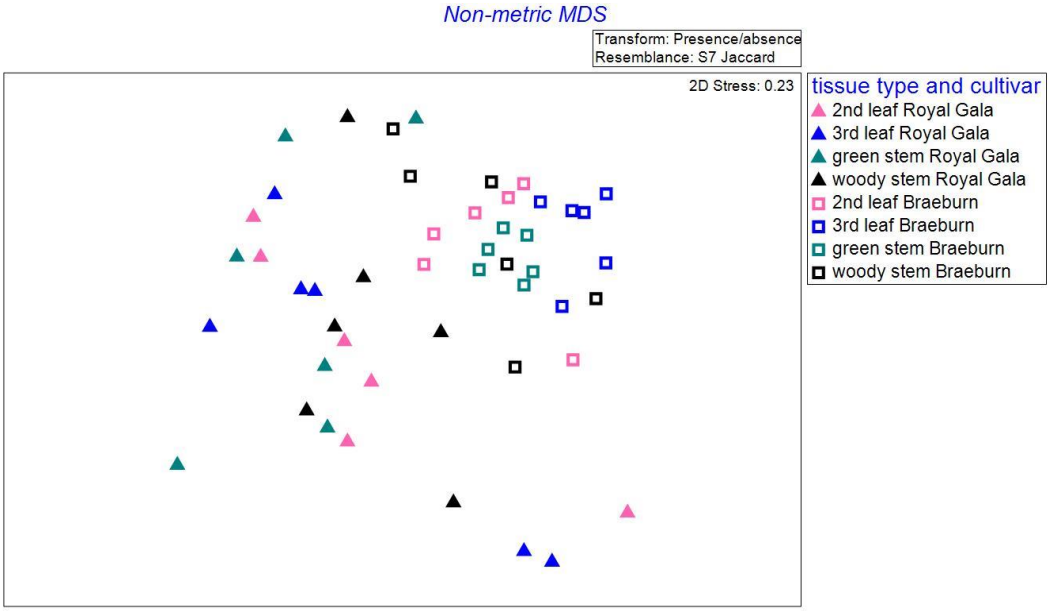

Figure 2.6 Nonmetric multidimensional scaling (nMDS) plot showing α-proteobacterial community in two cultivars 'Braeburn' (□), 'Royal Gala' (▲); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

Effect of tissue type and site on 'Royal Gala'

The interaction of tissue type and site significantly affected the α-proteobacterial communities (Table 2.7). The α-proteobacteria in tissues from site 4 and site 6 clustered by site, with those in samples from site 2 more dispersed than the other two sites (Figure 2.7, Appendix A2.13). There was no clustering by tissue type. Pairwise tests showed that the α-proteobacterial community in all tissue types from site 2 were not different (Appendix A2.14). For site 4 the α-proteobacterial communities were only different between the 2nd leaf and woody stem, while for site 6 the α-proteobacterial communities in the 2nd leaf differed from the other three tissue types. There was no difference in α-proteobacterial communities between the 3rd leaf, green stem and woody stem

for all the three sites. Pairwise tests showed the effect of site significantly influenced the α-proteobacterial community irrespective of tissue type.

α-proteobacterial richness was not affected by tissue type, site or their interaction (Table 2.7).

Table 2.7 Effect of tissue type and site on the similarity and richness of α-proteobacterial community identified using DGGE.

	α-proteobacteria	
Tissue type and site	Similarity[#]	Richness[$]
Tissue type	0.002**	0.123
Site	0.001**	0.616
Tissue type × site	0.003**	0.996

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

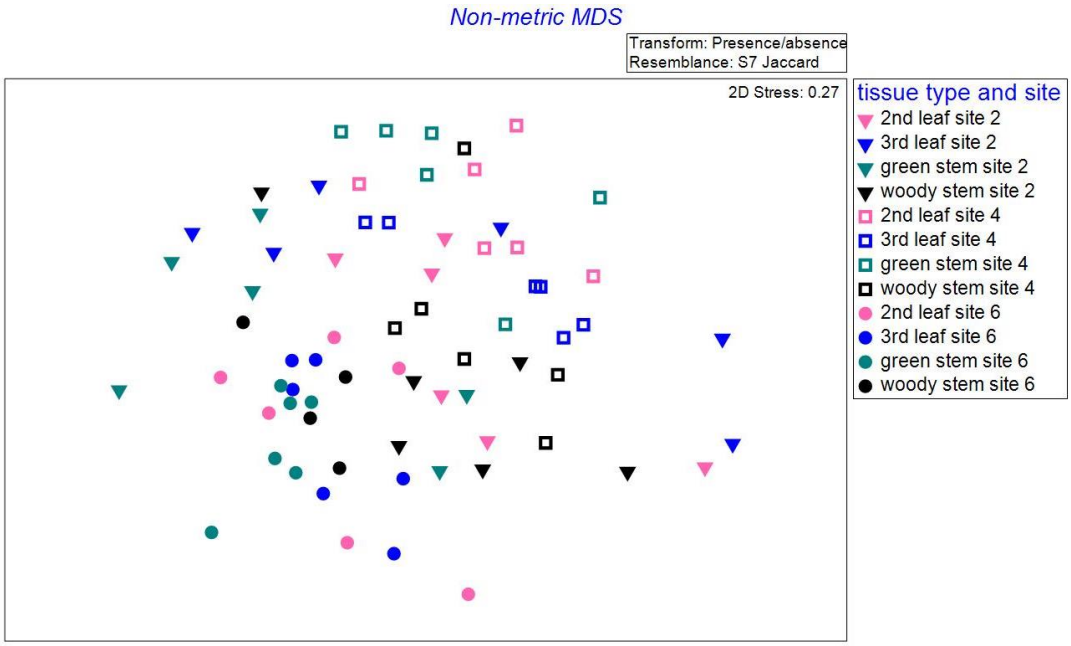

Figure 2.7 Nonmetric multidimensional scaling (nMDS) plot showing α-proteobacterial community from three sites; site 2 (▼), site 4 (□) and site 6 (●); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

2.3.1.4 β-proteobacteria

Tissue type and cultivar

Only cultivar significantly affected the β-proteobacterial community (Table 2.8). The β-proteobacterial community in each cultivar clustered, with 'Braeburn' more homogenous than 'Royal Gala' (Figure 2.8, Appendix A2.15). There was no cluster by tissue type in either cultivar.

β-proteobacterial richness was only significantly affected by cultivar (Table 2.8), with higher richness in 'Royal Gala' than in 'Braeburn' (Appendix A2.16).

Table 2.8 Effect of tissue type and cultivar on the similarity and richness of β-proteobacterial community identified using DGGE.

	β-proteobacteria	
Tissue type and cultivar	Similarity[#]	Richness[$]
Tissue type	0.119	0.197
Cultivar	0.001**	0.009*
Tissue type × cultivar	0.417	0.966

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

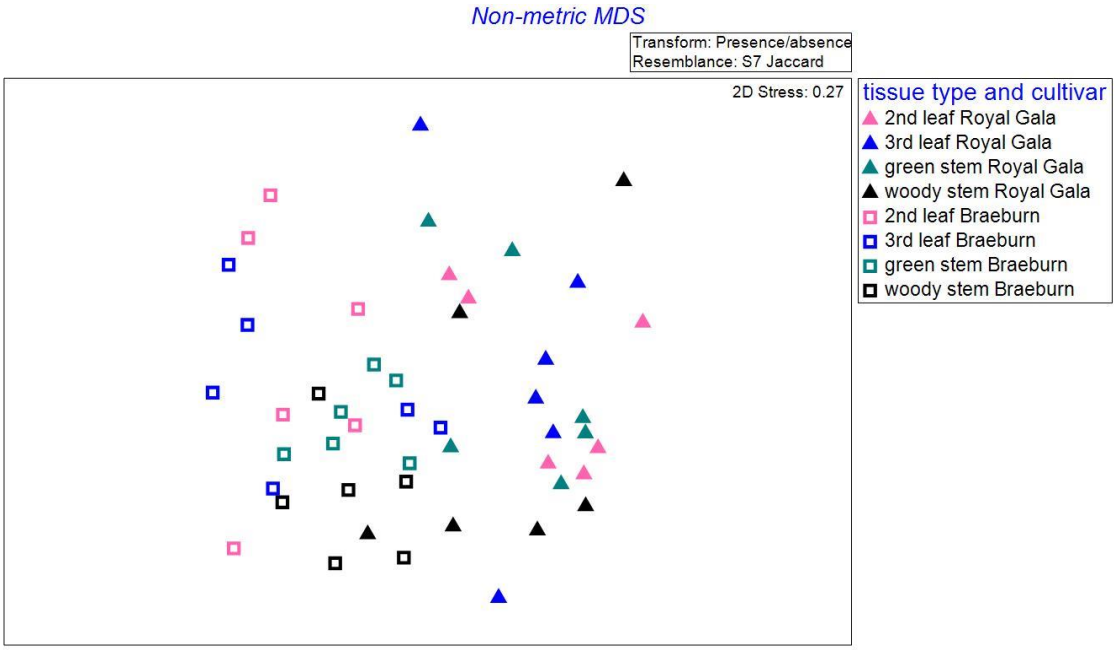

Figure 2.8 Nonmetric multidimensional scaling (nMDS) plot showing β-proteobacterial community in two cultivars 'Braeburn' (□), 'Royal Gala' (▲); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

The effect of tissue type and site on '*Royal Gala*'

The interaction of tissue type and site significantly affected β-proteobacterial communities (Table 2.9). The β-proteobacterial community from each site clustered together, with tissue samples for site 4 and site 6 more homogenous than those from site 2 (Figure 2.9, Appendix A2.17). They did not cluster by tissue type. Pairwise tests showed that for the 2nd leaf, 3rd leaf and green stem, β-proteobacterial communities were significantly different between all sites, while for woody stems there was only differences between sites 4 and 6 (Appendix A2.18). Pairwise tests showed tissue type did not affect the β-proteobacterial community in samples from site 2. In contrast, for site 4, the β-proteobacterial community in the 2nd leaf was significantly different from that in the other three tissues, with the 3rd leaf, green stem and woody stem similar to each other. For site 6, the β-proteobacterial community in the 2nd leaf was significantly different from that in the two stem tissues, with no difference between leaf tissues or between the 3rd leaf and either stem tissues (Appendix A2.18).

β-proteobacterial richness was only affected by site (Table 2.9). Pairwise tests showed the β-proteobacteria community in site 6 was richer than that in sites 2 and 4, with no difference between sites 2 and 4 (Appendix A2.18).

Table 2.9 Effect of tissue type and site on the similarity and richness of β-proteobacterial community identified using DGGE.

	β-proteobacteria	
Tissue type and site	Similarity#	Richness$
Tissue type	0.081	0.299
Site	0.001**	< 0.001**
Tissue type × site	0.004**	0.801

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

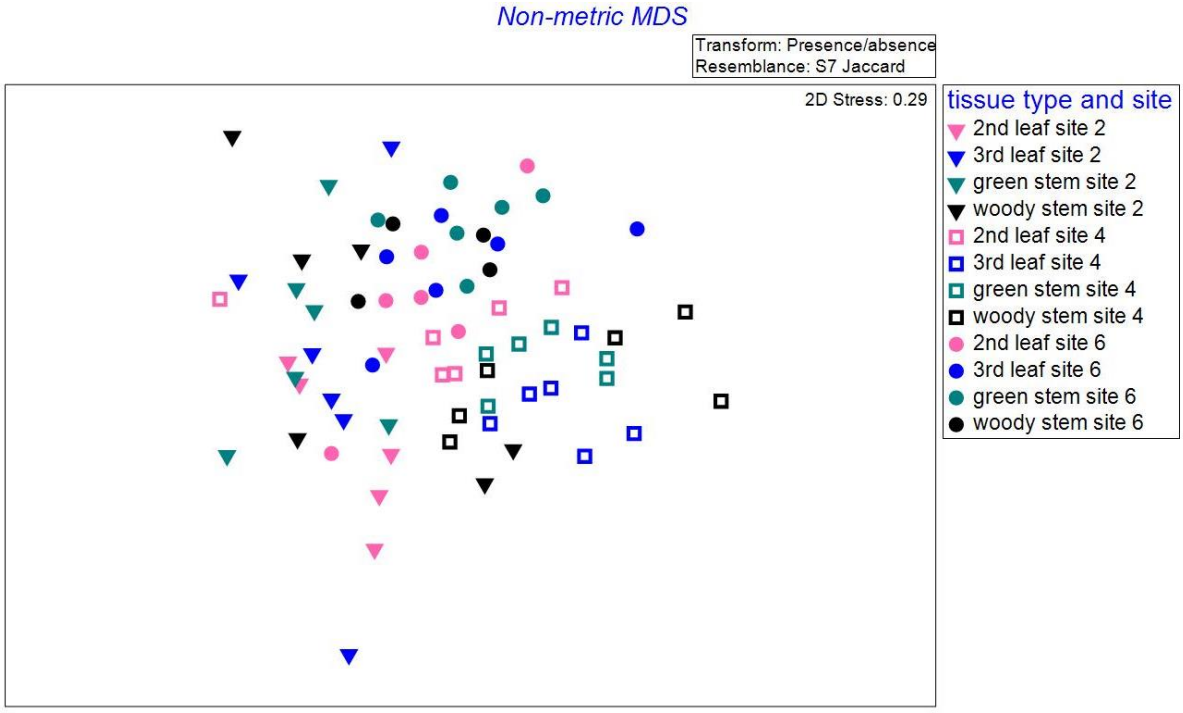

Figure 2.9 Nonmetric multidimensional scaling (nMDS) plot showing β-proteobacterial community from three sites; site 2 (▼), site 4 (□) and site 6 (●); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

2.3.1.5 γ-proteobacteria

Tissue type and cultivar

The interaction of tissue type and cultivar significantly affected the γ-proteobacterial community (Table 2.10). The γ-proteobacteria in each cultivar clustered together, and were more homogenous in 'Braeburn' than in 'Royal Gala' (Figure 2.10, Appendix A2.19). They did not cluster by tissue type. Pairwise tests showed that cultivar significantly affected the γ-proteobacterial community irrespective of tissue type (Appendix A2.20). Pairwise tests showed that for 'Royal Gala' there was no difference between the two leaf tissues, but the green stem was different to the woody stem. The 2nd leaf of 'Royal Gala' was also significantly different from green and woody stem. The same pattern was found for 'Braeburn', except that there was no different between the two stem tissues (Appendix A2.20).

Only cultivar significantly affected γ-proteobacterial richness (Table 2.10), with higher richness in 'Braeburn' than in 'Royal Gala' (Appendix A2.20).

Table 2.10 Effect of tissue type and cultivar on the similarity and richness of γ-proteobacterial community identified using DGGE.

	γ-proteobacteria	
Tissue type and cultivar	Similarity[#]	Richness[$]
Tissue type	0.004**	0.396
Cultivar	0.001**	0.012*
Tissue type × cultivar	0.002**	0.092

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

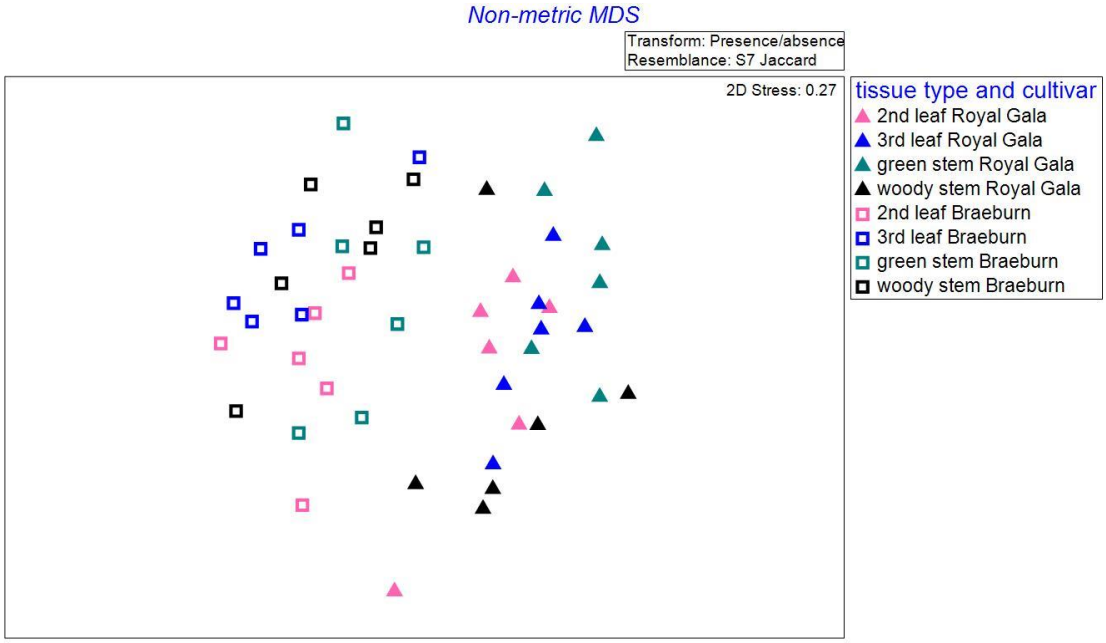

Figure 2.10 Nonmetric multidimensional scaling (nMDS) plot showing γ-proteobacterial community in two cultivars 'Braeburn' (☐), 'Royal Gala' (▲); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

The effect of tissue type and site on 'Royal Gala'

The interaction of tissue type and site significantly affected the γ-proteobacterial community (Table 2.11). The γ-proteobacterial community in samples from sites 4 and 6 clustered together (Figure 2.11, Appendix A2.21), with the γ-proteobacterial community in samples from site 2 being more dispersed. They did not cluster by tissue type.

Pairwise tests showed site had a significant effect on the γ-proteobacterial community in each tissue type, except between sites 4 and 6 in the 2nd leaf (Appendix A2.22). Pairwise tests showed that, for site 2, there was no difference in γ-proteobacterial community between the two leaf tissues. The γ-proteobacterial community in the 2nd leaf differed from green and woody stems,

while 3rd leaf was not different from the stem tissues. Also, in site 2 γ-proteobacterial communities in the two stem tissues differed. For site 4, γ-proteobacterial community only differed between the 3rd leaf and woody stem. In contrast, for site 6, the γ-proteobacterial community was only different between the green stem and each leaf tissue (Appendix A2.22).

γ-proteobacterial richness was not affected by tissue type, site or their interaction (Table 2.11).

Table 2.11 Effect of tissue type and site on the similarity and richness of γ-proteobacterial community identified using DGGE.

	γ-proteobacteria	
Tissue type and site	Similarity#	Richness$
Tissue type	0.005**	0.438
Site	0.001**	0.356
Tissue type × site	0.001**	0.079

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

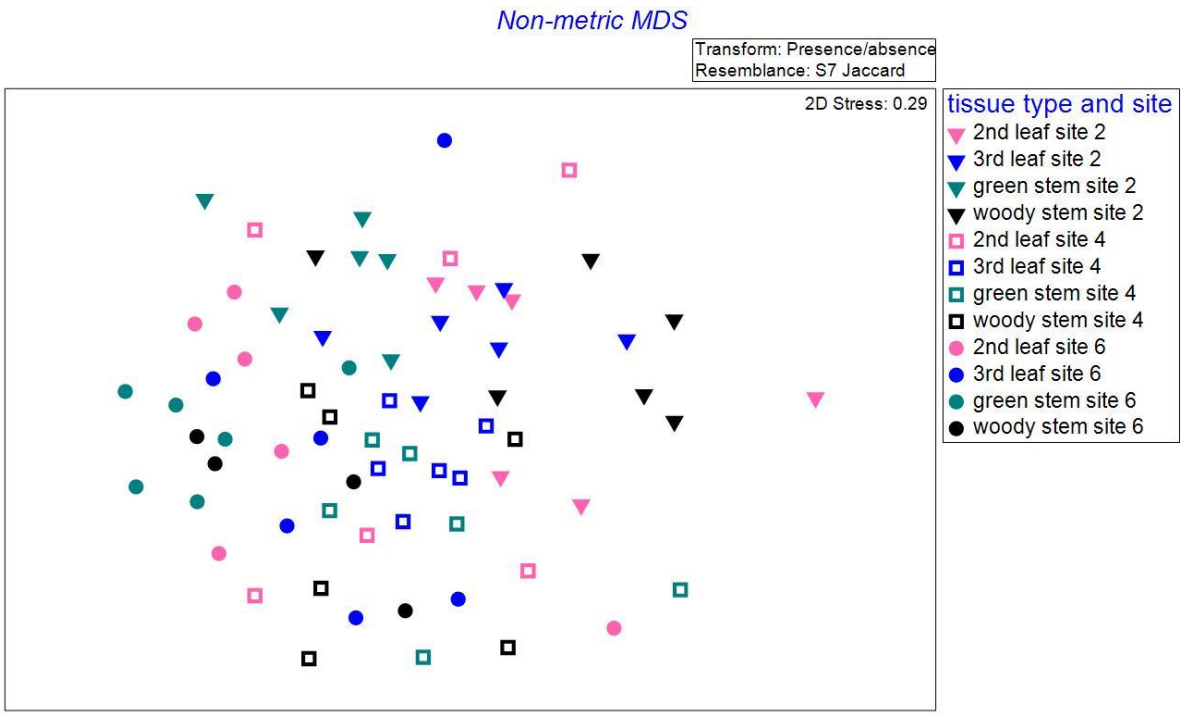

Figure 2.11 Nonmetric multidimensional scaling (nMDS) plot showing γ-proteobacterial community from three sites; site 2 (▼), site 4 (□) and site 6 (●); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

2.3.1.6 Actinobacteria

Tissue type and cultivar

The interaction of tissue type and cultivar significantly affected the actinobacterial community (Table 2.12). Actinobacterial communities in each cultivar clustered, with 'Braeburn' more homogenous than 'Royal Gala' (Figure 2.12, Appendix A2.23). They did not cluster by tissue type. Pairwise tests showed that the actinobacterial communities were significantly different between 'Royal Gala' and 'Braeburn' for all tissue types (Appendix A2.24). Pairwise tests showed that, for 'Royal Gala', the actinobacterial community in the 2nd leaf was different from that in the other three tissues, with no difference between any other tissue types. For 'Braeburn', only the actinobacterial community in woody stem differed from that in the leaf tissues (Appendix A2.24).

Tissue type and cultivar significantly affected actinobacterial richness, but there was no interaction between them (Table 2.12). Pairwise tests showed that actinobacteria in the leaf tissues were similar to each other and richer than that in the woody stem. Green stem did not differ in richness to the other three tissues (Appendix A2.24). Pairwise tests showed that actinobacterial community in 'Royal Gala' was richer than that in 'Braeburn' (Appendix A2.24).

Table 2.12 Effect of tissue type and cultivar on the similarity and richness of actinobacterial community identified using DGGE.

	Actinobacteria	
Tissue type and cultivar	Similarity[#]	Richness[$]
Tissue type	0.002**	0.050*
Cultivar	0.001**	< 0.001**
Tissue type × cultivar	0.002**	0.153

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

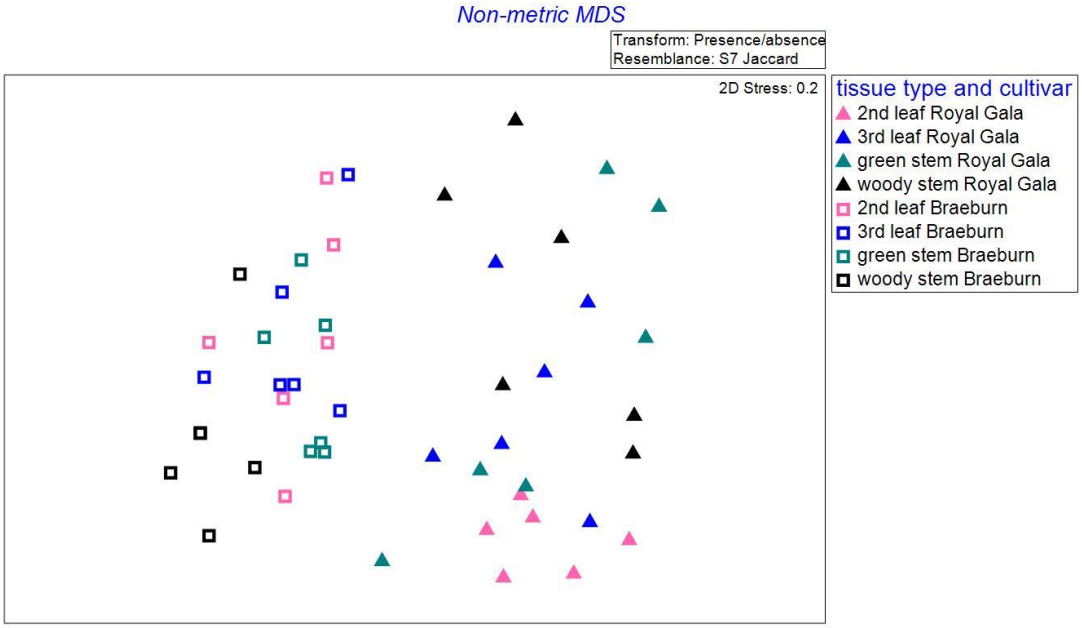

Figure 2.12 Nonmetric multidimensional scaling (nMDS) plot showing actinobacterial community in two cultivars 'Braeburn' (□), 'Royal Gala' (▲); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

The effect of tissue type and site in 'Royal Gala'

The interaction of tissue type and site significantly affected the actinobacterial communities (Table 2.13). The actinobacterial community in each site clustered, with samples from site 6 the most homogenous (Figure 2.13, Appendix A2.25). They did not cluster by tissue type. Pairwise tests showed that site affected the actinobacterial community irrespective of tissue type (Appendix A2.26). Pairwise tests showed that for all three sites, the actinobacterial community in the 2nd leaf differed from that in the other three tissue types (Appendix A2.26). In addition, for site 2, there was no difference between the 3rd leaf, green stem and woody stem. In contrast, for site 4, the actinobacterial community in the 3rd leaf differed from woody but not green stems. For site 6, the actinobacterial community in the 3rd leaf differed from green but not woody stems. There was no difference in the actinobacterial community between two stem tissues from sites 4 and 6 (Appendix A2.26).

Tissue type and site significantly affected actinobacterial richness, but there was no interaction between them (Table 2.13). Pairwise tests showed that there was no difference in the actinobacterial community richness between the two leaf tissues or between the two stem tissues (Appendix A2.26). In addition, the actinobacterial community was richer in the 2nd leaf than the two stem tissues. Actinobacterial community in the 3rd leaf was richer than that in woody stem but not

the green stem. Pairwise tests showed that actinobacteria were richest in site 4, with site 2 the least rich (Appendix A2.26).

Table 2.13 Effect of tissue type and site on the similarity and richness of actinobacterial community identified using DGGE.

	Actinobacteria	
Tissue type and site	Similarity[#]	Richness[$]
Tissue type	0.001**	0.001**
Site	0.001**	< 0.001**
Tissue type × site	0.001**	0.057

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

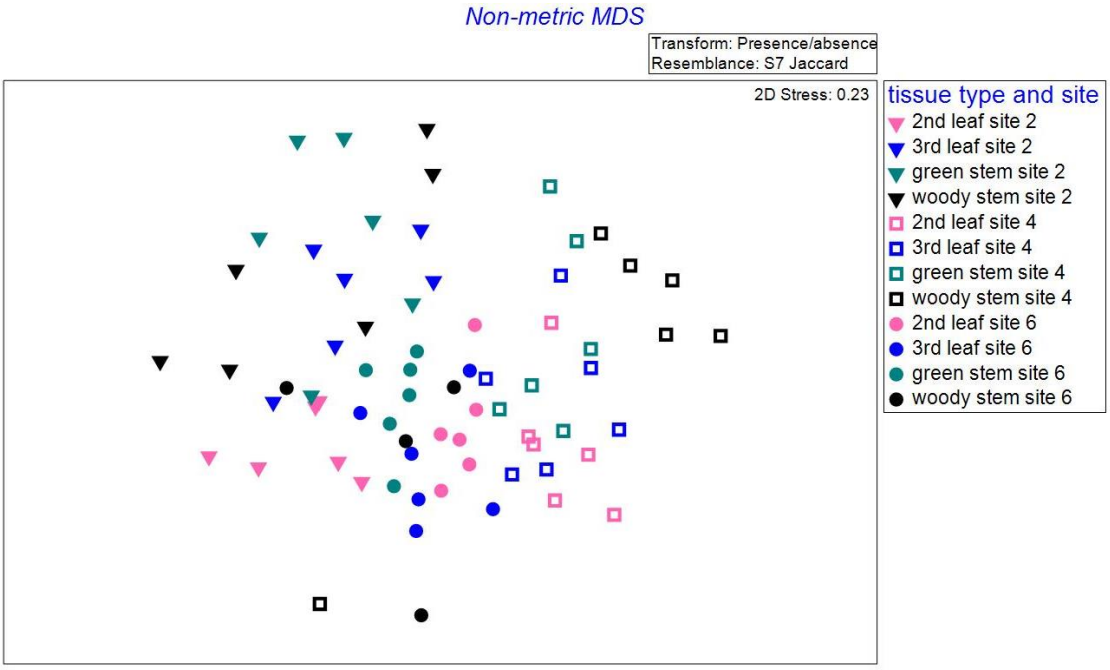

Figure 2.13 Nonmetric multidimensional scaling (nMDS) plot showing actinobacterial community from three sites; site 2 (▼), site 4 (□) and site 6 (●); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black).

2.3.2 Effect of region, season and cultivar on endophyte communities

As tissue type, cultivar and site significantly affected endophyte communities (Section 2.3.1), this analysis was not repeated in this section. As *N. ditissima* is a woody pathogen, tissue samples and the community analysis was restricted to woody stems in this section. Endophyte communities were reduced to fewer groups, with the focus on total bacteria, total fungi, γ-proteobacteria and

actinobacteria. This is because these were variable in Section 2.3.1 and because *Pseudomonas* isolates selected as biocontrol candidates in Chapter 3 were γ-proteobacteria. Actinobacteria were assessed, as this taxa is known to be bioactive and only one was isolated in Chapter 3, despite large unculturable communities being present. Available data allowed main tests for region (sites 5, 6 in Hawke's Bay vs sites 1, 4 in Nelson) and season (at site 1, 2 and 4, respectively) to be done. Extension of sampling to the woody stems of cultivar 'Scifresh' allowed cultivar to be reassessed at a single site (site 2 and 6, respectively) across three cultivars, but for woody stems only.

2.3.2.1 Total bacteria

Region did not affect the total bacterial community in woody stems of either 'Royal Gala' or 'Braeburn' (Table 2.14), but there was a significant variation between sites. The total bacterial richness in woody stems of 'Braeburn' was significantly affected by region (Table 2.14), with communities in Nelson (n = 10.8) richer than in Hawke's Bay (n = 7.5). There was no effect of region on 'Royal Gala'.

Season significantly affected the total bacterial community in woody stems of 'Royal Gala' at all three sites and 'Braeburn' in site 1 (Table 2.15). Total bacterial richness was not affected by season (Table 2.15).

Cultivar did not affect the total bacterial community and richness in woody stems at either site (Table 2.16).

Table 2.14 Effect of region on the similarity and richness of total bacterial community identified using DGGE.

		Total bacteria	
Effect of region in each cultivar		Similarity[#]	Richness[$]
'Royal Gala'	Region	0.448	0.196
	Site (nested in region)	0.005**	-
'Braeburn'	Region	0.149	< 0.001**
	Site (nested in region)	0.007*	-

- means not tested. # denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Table 2.15 Effect of season on the similarity and richness of total bacterial community identified using DGGE.

		Total bacteria	
Effect of season in each cultivar across three sites		Similarity[#]	Richness[$]
Site 1	'Royal Gala'	0.034*	0.422
	'Braeburn'	0.007*	0.070
Site 2	'Royal Gala'	0.016*	0.643
	'Braeburn'	0.126	0.820
Site 4	'Royal Gala'	0.006*	0.374
	'Braeburn'	0.288	0.417

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Table 2.16 Effect of cultivar on the similarity and richness of total bacterial community identified using DGGE.

	Total bacteria	
Effect of cultivar across two sites	Similarity[#]	Richness[$]
Site 2	0.055	0.317
Site 6	0.386	0.670

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM).

2.3.2.2 Total fungi

The total fungal community and community richness in woody stems of 'Royal Gala' and 'Braeburn' were not affected by region (Table 2.17). There was variation between sites.

Season affected the total fungal community in woody stems of 'Braeburn' at site 2 (Table 2.18) but did not affect communities in 'Royal Gala'. Total fungal richness in woody stems of 'Royal Gala' and 'Braeburn' at the three sites was not affected by season (Table 2.18).

The total fungal community was significantly affected by cultivar at both sites (Table 2.19). There was no difference between cultivars at site 2, but the total fungal community in 'Braeburn' differed from 'Scifresh' at site 6 (Table 2.20). The richness of the total fungal community was significantly affected by cultivar at site 6 but not site 2 (Table 2.19), with 'Royal Gala' (n = 9.0) and 'Scifresh' (n = 9.0) being richer than 'Braeburn' (n = 5.0) (Table 2.20).

Table 2.17 Effect of region on the similarity and richness of total fungal community identified using DGGE.

		Total fungi	
Effect of region in each cultivar		Similarity[#]	Richness[$]
'Royal Gala'	Region	0.567	0.727
	Site (nested in region)	0.001**	-
'Braeburn'	Region	0.787	0.210
	Site (nested in region)	0.013*	-

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Table 2.18 Effect of season on the similarity and richness of total fungal community identified using DGGE.

		Total fungi	
Effect of season in each cultivar across three sites		Similarity[#]	Richness[$]
Site 1	'Royal Gala'	0.390	0.154
	'Braeburn'	0.331	0.073
Site 2	'Royal Gala'	0.189	0.718
	'Braeburn'	0.038*	0.335
Site 4	'Royal Gala'	0.157	0.155
	'Braeburn'	0.278	0.387

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$).

Table 2.19 Effect of cultivar on the similarity and richness of total fungal community identified using DGGE.

	Total fungi	
Effect of cultivar across two sites	Similarity[#]	Richness[$]
Site 2	0.034*	0.237
Site 6	0.020*	0.047*

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$).

Table 2.20 Pairwise comparison of total fungal community similarity and richness in woody stem of 'Royal Gala', 'Braeburn' and 'Scifresh' from site 2, site 4 and site 6. Mean of richness of actinobacterial community was in the bracket.

Pairwise test of total fungal similarity		
Site 2	'*Braeburn*'	'*Scifresh*'
'*Royal Gala*'	0.158	0.078
'*Braeburn*'		0.083
Site 6	'*Braeburn*'	'*Scifresh*'
'*Royal Gala*'	0.108	0.105
'*Braeburn*'		0.038*
Pairwise test of total fungal richness		
Site 6	'*Braeburn*' *(5.0)*	'*Scifresh*' *(9.0)*
'*Royal Gala*' *(9.0)*	0.030*	1.000
'*Braeburn*' *(5.0)*		0.030*

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$).

2.3.2.3 γ-proteobacteria

Region did not affect the γ-proteobacterial community in woody stems of either 'Royal Gala' or 'Braeburn' (Table 2.21). There was variation between sites. The γ-proteobacterial richness in woody stems of 'Royal Gala', but not 'Braeburn', was significantly affected by region (Table 2.21), with higher richness in Hawke's Bay (n = 13.3) than in Nelson (n = 9.3).

Season did not affect the γ-proteobacterial community in woody stems of 'Royal Gala' at any site (Table 2.22). For 'Braeburn', season only affected the γ-proteobacterial community at site 2. Season only affected γ-proteobacterial richness in woody stems of 'Royal Gala' at site 2, and 'Braeburn' at site 4 (Table 2.22). For both cultivars, γ-proteobacteria was richer in autumn than in spring ('Royal Gala' at site 2: autumn = 9.7, spring = 4.7; 'Braeburn' at site 4: autumn = 14.7, spring = 7.7).

Cultivar significantly affected the γ-proteobacterial community at both sites (Table 2.23), with 'Royal Gala' significantly different from 'Scifresh' at both sites (Table 2.24). At site 2, 'Royal Gala' was also different from 'Braeburn'. The γ-proteobacterial richness was significantly affected by cultivar at site 2 but not at site 6 (Table 2.23), with 'Royal Gala' significantly less rich than 'Braeburn' and 'Scifresh' (Table 2.24).

Table 2.21 Effect of region on the similarity and richness of γ-proteobacterial community identified using DGGE.

		γ-proteobacteria	
Effect of region in each cultivar		Similarity[#]	Richness[$]
'Royal Gala'	Region	0.511	0.035*
	Site (nested in region)	0.001**	-
'Braeburn'	Region	0.354	0.137
	Site (nested in region)	0.001**	-

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Table 2.22 Effect of season on the similarity and richness of γ-proteobacterial community identified using DGGE.

		γ-proteobacteria	
Effect of season in each cultivar across three sites		Similarity[#]	Richness[$]
Site 1	'Royal Gala'	0.097	0.265
	'Braeburn'	0.125	0.541
Site 2	'Royal Gala'	0.113	0.006*
	'Braeburn'	0.032*	0.320
Site 4	'Royal Gala'	0.135	0.187
	'Braeburn'	0.179	0.002**

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Table 2.23 Effect of cultivar on the similarity and richness of γ-proteobacterial community identified using DGGE.

	γ-proteobacteria	
Effect of cultivar across two sites	Similarity[#]	Richness[$]
Site 2	0.004*	0.022*
Site 6	0.044*	0.129

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$).

Table 2.24 Pairwise comparison of γ-proteobacterial community similarity and richness in woody stem of 'Royal Gala', 'Braeburn' and 'Scifresh' from site 2, site 4 and site 6. Mean of richness of actinobacterial community was in the bracket.

Pairwise test of γ-proteobacterial similarity		
Site 2	'*Braeburn*'	'*Scifresh*'
'*Royal Gala*'	0.018*	0.042*
'*Braeburn*'		0.191
Site 6	'*Braeburn*'	'*Scifresh*'
'*Royal Gala*'	0.126	0.046*
'*Braeburn*'		0.244
Pairwise test of γ-proteobacterial richness		
Site 2	'*Braeburn*' *(n = 16.7)*	'*Scifresh*' *(n = 15.0)*
'*Royal Gala*' *(n = 4.7)*	0.011*	0.021*
'*Braeburn*' *(n = 16.7)*		0.634

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$).

2.3.2.4 Actinobacteria

Region did not affect the actinobacterial community and richness in woody stems of 'Royal Gala' and 'Braeburn' (Table 2.25). There was variation between sites only for 'Braeburn'.

Season only affected the actinobacterial community and richness in woody stems of 'Braeburn' at site 1 (Table 2.26), with autumn having less actinobacterial richness than spring (spring = 16.0, autumn = 10.7).

Cultivar did not affect the actinobacterial community or community richness at either site (Table 2.27).

Table 2.25 Effect of region on the similarity and richness of actinobacterial community identified using DGGE.

		Actinobacteria	
Effect of region in each cultivar		Similarity[#]	Richness[$]
'Royal Gala'	Region	0.395	0.194
	Site (nested in region)	0.281	-
'Braeburn'	Region	0.080	0.311
	Site (nested in region)	0.048*	-

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$).

Table 2.26 Effect of season on the similarity and richness of actinobacterial community identified using DGGE.

		Actinobacteria	
Effect of season in each cultivar across three sites		Similarity[#]	Richness[$]
Site 1	'Royal Gala'	0.065	0.492
	'Braeburn'	0.022*	0.001**
Site 2	'Royal Gala'	0.229	0.111
	'Braeburn'	0.117	0.279
Site 4	'Royal Gala'	0.462	0.451
	'Braeburn'	0.101	0.917

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Table 2.27 Effect of cultivar on the similarity and richness of actinobacterial community identified using DGGE.

	Actinobacteria	
Effect of cultivar across two sites	Similarity[#]	Richness[$]
Site 2	0.128	0.171
Site 6	0.255	0.417

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM).

2.4 Discussion

This is the first study to characterise the structure of the complete microbial endophyte community (culturable and unculturable) in apple leaf and stem tissues as affected by different orchard factors including tissue type, season, region, site and cultivar in New Zealand. This was achieved by using the culture-independent method DGGE to characterise different groups of taxa, including total bacteria, total fungi, proteobacteria (α-, β-, γ-proteobacteria) and actinobacteria, and established their relationship with tissue type, site and cultivar. Proteobacteria were investigated in detail because they are reported as the dominant group of endophytes in woody plants such as poplar (*Populus deltoides*) and apple (Gottel et al., 2011; Liu et al., 2018). Additionally, the various proteobacteria (α-, β-, γ-proteobacteria) and actinobacteria are known to be beneficial to plants and may have biocontrol potential (Bruto et al., 2014; Purushotham et al., 2018). For example, *Sphingomonas* spp. belonging to the α-proteobacteria suppressed disease symptoms and reduced growth of the leaf pathogen *Pseudomonas syringae* pv. *tomato* on

Arabidopsis thaliana under experimental conditions (Innerebner et al., 2011). When investigating the effect of region and season, the experiments focused on γ-proteobacteria and actinobacteria because *Pseudomonas* spp. (γ-proteobacteria) isolates were identified as potential biocontrol agents in this study, and the low recovery of actinobacteria into culture in this study meant that the significance of this taxa was not well explored (single actinobacterium isolated; Chapter 3).

Tissue type typically influenced the bacterial and fungal endophyte communities assessed in this study, in both 'Royal Gala' and 'Braeburn', with the exception of the β-proteobacteria. The effect of tissue type on endophytic bacterial communities has been reported previously (Campisano et al., 2017; Purushotham Balraj, 2017; Sessitsch et al., 2002; Wicaksono et al., 2016). Purushotham Balraj (2017) also found plant tissues significantly affected the total fungal community in horopito using DGGE. In contrast, using DGGE Wicaksono et al. (2016) and Purushotham Balraj (2017) both found that tissue type affected α- and β-proteobacteria communities in two New Zealand medicinal plants, mānuka (*Leptospermum scoparium*) and horopito (*Pseudowintera colorata*), respectively, but did not find any difference in γ-proteobacteria. A possible reason could be differences in key bacterial communities between domesticated plants and native medicinal plants, with the absence of potent antimicrobial compounds (e.g. essential oils, polygodial) in apple. The process of domestication can impact the composition of the plant-associated microbiota, due to the coevolution of host-microbe interactions (Pérez-Jaramillo et al., 2018). Another reason may be that more tissues, including roots, were investigated in their studies which may have allowed more proteobacteria taxa to be evaluated. Robinson et al. (2016) reported proteobacteria were the most prevalent culturable endophytes in roots as compared to that in leaves. Peršoh (2013) reported both host species and tissue types were major factors shaping endophytic fungal communities in leaf and stem of *Viscum album* and *Pinus sylvestris*.

Tissue type influenced the richness of the total bacterial and actinobacterial communities in both 'Royal Gala' and 'Braeburn', but this was not observed for the proteobacterial (α-, β-, γ-proteobacterial) and total fungal community richness. In contrast, Wicaksono et al. (2016) found plant tissue (leaf, stem and root) affected total bacterial, and α-, γ-proteobacterial richness in mānuka (*Leptospermum scoparium*) using DGGE. This could be because the different plant host and more tissue types were included in their study as was discussed previously. The total bacterial and actinobacterial community richness varied in different tissue types, with 2nd leaf the least rich for total bacteria and with richness of actinobacteria in leaves higher than that in woody stems. Variation in richness of different endophyte taxa in plant tissues was also found by Wicaksono et al. (2016), however, conversely they found a higher richness in leaf for total bacteria. In contrast, species richness of culturable endophytic fungi was found to be different

between trunk and leaf of *Citrus reticulate* (Sadeghi et al., 2019) and between leaf and stem tissues of *Azadirachta indica* (Neem) from India (Verma et al., 2007). The different results compared with the previous studies could be attributed to various factors such as the different plant species and different sites which also contributed to the shaping of fungal endophyte community in plant tissues (Martín-García et al., 2011; Raviraja, 2005; Sun et al., 2012).

This study demonstrated that bacterial and fungal endophyte communities (except β-proteobacteria) in leaves often differed from those in stems in 'Royal Gala' and 'Braeburn'. Similarly, da Silva et al. (2013) found total bacterial community in leaf and stem of pepper-rosmarin (*Lippia sidoides*) were different. Win et al. (2018) reported that culturable endophytic fungal community in stem tissues of three Japanese tea cultivars (*Camellia sinensis)* were more diverse than that in leaf tissues. A similar result was reported by Sadeghi et al. (2019) that stems and leaves of *Citrus reticulate* had different diversity of culturable fungal endophytes and core fungal taxa, with stems habouring more diversity than leaves. In contrast, Jin et al. (2014) found no significant difference in the endophytic bacterial community of leaf and stem samples of the medicinal plant *Stellera chamaejasme*. This could be because Jin et al. (2014) compared the bacterial community of three habitats including rhizosphere, leaf and stem, and found there was a significant difference between rhizosphere samples and plant samples. As the rhizosphere was highly diverse the analysis showed the leaf and stem samples were more similar to each other. PMA treatment for excluding epiphytic DNA to be amplified by PCR was not mentioned in the study of da Silva et al. (2013) and Jin et al. (2014). This could affect the result of endophyte community evaluation. Another possible reason for the difference in the bacterial community structure between leaf and stem samples found in this study could be due to the varied migration ability of bacterial endophytes inside the plants. Chi et al. (2005) reported that the bacterial community in leaf tissue could accumulate endophytes by ascending endophytic migration from below-ground to above-ground tissues. However, this would only be a subset of bacteria due to the varied migration ability. Compant et al. (2005) found some bacteria in the rhizosphere of grapevine could reach the leaves in 84 h. Moreover, leaves are known to acquire airborne bacteria and fungi from the immediate environment (Bulgarelli et al., 2013; Herre et al., 2007). It may contribute to the difference of endophyte community between leaves and stems.

The two leaf ages (approx. 2 weeks old vs. 4-6 weeks old) showed similarity in total bacterial and total fungal communities within each cultivar. This indicated that bacterial and fungal endophytes were not affected by the leaf ages tested. Similarly, Ercolani (1991) reported leaves of different ages were more similar in bacterial community when sampled at the same time (as this study) than leaves of the same age sampled at a different time. Arnold and Herre (2003) found leaf age

affected fungal endophytes due to the duration of exposure to aerial and epiphytic fungi but not the absolute leaf age. Therefore, the similarity in total bacterial and fungal communities in the two leaf tissues could be due to the reasons previously discussed that leaves acquire airborne bacteria and fungi from the immediate environment (Bulgarelli et al., 2013; Herre et al., 2007). During this period, the external microbial community which colonises the leaf tissue may remain relatively constant, contributing to the similarity of endophyte community between the two leaves. Total fungal community, but not total bacteria, was also similar between two stem tissues (approx. 2 months old vs. 3 months old), indicating the fungal community was less affected by stem age. Peršoh (2013) found that the endophytic fungal community was different between 1- and 3-year old stem sections of *Viscum* and *Pinus*, but the fungal community in 2-year old stem was indistinguishable from the younger and older stems. Therefore, the similarity in total fungal community in the two stem tissues in this study could be because age difference was small. This can also be a possible reason for the similar endophyte community in the two leaves used in this study.

Cultivar was a significant factor influencing all the bacterial and fungal endophyte communities, with the endophyte taxa in 'Royal Gala' more variable than that in 'Braeburn'. Difference of endophytic fungal diversity in stems was also found between three apple cultivars ('Golden Delicious', 'Honey Crisp' and 'Royal Gala') by Liu et al. (2018). Plant genotype at a cultivar level may be a determinant factor in the shaping of endophyte community, which was reported in other plant species such as potato (*Solanum tuberosum*) (Andreote et al., 2010) and grapevine (*Vitis vinifera*) (Jayawardena et al., 2018). It can be explained by a selection mechanism from plant genotype, which allowed cultivars to filter microbiomes as their endophytes from the environment (Whipps et al., 2008). The different endophyte community in the two cultivars could also be because of the difference in their parentages. 'Royal Gala' is a sport of 'Gala' crossed by 'Kidd's Orange Red' and 'Golden Delicious' and 'Braeburn' is a cross of 'Lady Hamilton' and 'Granny Smith'. Liu et al. (2018) found that cultivars with closer pedigree had more similar microbial communities than those with distantly-related pedigrees. Further, Liu et al. (2018) suggested that apple rootstock/scion cultivar combinations affected the composition of fungal endophytic community. As both of the scion cultivars were grafted on the same rootstock 'MM.106', genotype of rootstock could not be the reason for the difference in the endophyte community between them. However, endophyte community in apple trees maybe affected by the site of nursery they were sourced from, as site was found to be a significant factor in shaping endophyte community in apple tissues by this study. Therefore, the site of nursery may have determined the initial endophyte community in the grafted apple trees prior to these being

planted in the orchards. 'Royal Gala' had a higher richness of β-proteobacterial and actinobacterial communities than 'Braeburn', while 'Braeburn' was richer in total bacterial and γ-proteobacterial communities. The factor of cultivar is an important factor influencing species richness of endophyte communities in other plants such as rice and grapevine (Edwards et al., 2015; Jayawardena et al., 2018). This result reinforced the need to isolate endophytes from different cultivars for selecting potential biocontrol agents in Chapter 3.

The extension of the assessment of cultivar effects to include 'Scifresh' showed total fungal and γ-proteobacterial communities were different between the three cultivars, but no difference was found for total bacterial and actinobacterial communities. A possible reason for this is that 'Scifresh' is a cross of 'Braeburn' and 'Royal Gala', and thus is intermediate between the two cultivars. Based on the rationale for the effect of parentage reported by Liu et al. (2018), the inclusion of 'Scifresh' in cultivar comparison reduced the parentage difference among cultivars. This may contribute to lowering the significant difference between endophyte communities. Furthermore, only woody stem was assessed in this experiment further reducing difference amongst the three cultivars. Additionally, the effect of cultivar on endophyte community may be associated with their resistance to European canker. The three cultivars in this study were selected based on their difference in European canker resistance, with 'Royal Gala' more resistant than 'Scifresh' and 'Braeburn' being unknown. Hirakue and Sugiyama (2018) showed that resistance of apple cultivars to Alternaria leaf spot (*Alternaria mali*) was related to the fungal endophyte diversity in leaves. Herre et al. (2007) found foliar endophytes, commonly present in healthy leaves of *Theobroma cacao*, improved host defences against foliar damage caused by *Phytophthora palmivora*. For this reason, the fungal and γ-proteobacterial taxa which were different between the resistant and susceptible cultivar may be important potential sources of biocontrol agents. Future work is recommended to further investigate the relationship of cultivar resistance to European canker and endophyte communities in apple tissues. This can be achieved by inoculation of *N. ditissima* on cultivars with different European canker resistance to evaluate the correlation between endophyte community and disease severity.

Site significantly affected all six bacterial and fungal endophyte communities in all tissues of 'Royal Gala', and also the richness of most endophyte taxa in several tissues. Martín-García et al. (2011) found that site affected the fungal endophyte community and richness in leaves and twigs from branches of poplar (*Populus* × *euramericana*). Knief et al. (2010) found that site had a strong impact on the *Methylobacterium* (belonging to α-proteobacteria) community composition that ubiquitously occur in leaves of various plant species, such as *Arabidopsis thaliana* and *Medicago truncatula*. Site also significantly affected the bacterial community structure of the wheat

rhizosphere, even though the two sites they sampled had similar soils and environments (Donn et al., 2015). Site comprises a complex range of factors such as soil and weather/climatic conditions, which could influence the microbial community structure surrounding plants (Costa et al., 2006; Edwards et al., 2015; Rasche et al., 2006). Soil conditions could affect the endophyte communities in leaf and stem tissues as taxa arising from the microbiomes within the rhizosphere could gain entry to the roots and migrate to above-ground tissues (Chi et al., 2005). Crop management practices influence soil conditions, resulting in an effect on soil bacterial community structure and thus root-endophytic fungi (Acosta-Martínez et al., 2008; Wilberforce et al., 2003). The three sites (site 2, site 4 and site 6) in the current study were all managed by IFP showed that overall management practices did not account for the differences in the endophyte communities. However, although all were managed by IFP, specific management practices related to the specific issues between orchard sites such as pest/pathogen problems or nutrients could account for differences. The communities of all six endophyte microbial taxa were different in site 2 (Hope, Nelson), as compared to site 4 (Riwaka, Nelson) and site 6 (Hawke's Bay), suggesting that region, which is associated with weather conditions, may not affect the endophyte community in this study. Therefore, it is more likely that soil type and microclimate contributed to the site effect.

As site influenced the endophyte communities in apple tissues, the analysis was extended to consider region as a factor affecting endophyte communities, focusing on total bacteria, total fungi, γ-proteobacteria and actinobacteria in woody stems. Region had no effect on the four test endophyte communities in both 'Royal Gala' and 'Braeburn', but did have some effect on richness. In contrast, other studies have shown endophytic fungi in leaves were affected by geographic location and this was because they are mainly horizontally transmitted through associations in the local environment (Christian et al., 2016; Saikkonen, 2007; Zimmerman & Vitousek, 2012). Parmar et al. (2018) also reported that location influenced total endophytic fungal community structure in roots and shoots of *Dysphania ambrosioides*. Using DGGE Purushotham Balraj (2017) and Wicaksono et al. (2016) found region affected bacterial and fungal endophyte communities of two medicinal plants horopito (*Pseudowintera colorata*) and mānuka (*Leptospermum scoparium*). The reason for the nonsignificant influence from region in the current study may be because site variation within the two regions was strong. In this study, the microbiome may be spread in each of the two regions independent on geographic location, but the endophytes in apple trees were determined by site related soil conditions in orchards and nursery, and cultivar as it was discussed previously. Rootstock used in the two regions were different, with 'MM.106' in Nelson and 'M.9' in Hawke's Bay, except for 'Braeburn' being grafted

by ‘MM.106’ at site 6 in Hawke’s Bay. Therefore, rootstock/scion combinations is probably not the main effect on endophyte community between the two regions.

Region affected the richness of total bacterial and γ-proteobacterial communities in woody stems, with higher total bacterial community richness in Nelson for ‘Braeburn’ and higher γ-proteobacterial community richness in Hawke’s Bay for ‘Royal Gala’. It suggested total bacterial and γ-proteobacterial richness in apple stems were more affected by environmental changes than local site differences. Bacterial species richness affected by geographic location was also reported on leaf surface of *Tamarix* (a salt-secreting desert tree) (Finkel et al., 2011). This could be attributed to the differing responses of leaves related to hosts (species and/or cultivars) to environmental variables, which determined whether microbial cells could colonise the leaf (Whipps et al., 2008). Therefore, the effect of region on the richness of total bacteria and γ-proteobacteria in apple stems could be because they migrated from leaf tissues, which obtained bacteria such as *Pseudomonas* (γ-proteobacteria) from the immediate environment (Bulgarelli et al., 2013).

Season generally affected total bacterial community but not total fungal, γ-proteobacterial and actinobacterial communities in woody stems across three sites in two cultivars, with typically no influence on richness of endophyte taxa. These results illustrated that season was not a major factor in the shaping of endophyte communities in woody stems of apple. In contrast, seasonal variation was reported as a determinant factor structuring the complete bacterial endophyte communities in branches of different tree species *Acer negundo* (Manitoba Maple)*, Ulmus pumila* (Siberian Elm) and *U. parvifolia* (Chinese Elm) collected from the same site, with effects on the abundance of actinobacteria and proteobacteria (Shen & Fulthorpe, 2015). The different result in this study could be due to only woody stem being tested, while the study of Shen and Fulthorpe (2015) included both green and woody stems, as defined in this study. Season has been shown to affect colonisation and/or isolation rate of endophytes in other plant species due to the variations in temperature (Mocali et al., 2003), wetness period (Rather et al., 2018), as well as growth stage of the tissue (Guo et al., 2008). As woody stems are more stable and also lignified as compared with leaves and green stem, the endophyte community in woody stems maybe more consistent during the year. Further, the endophyte community in the woody stem may be mainly acquired from the underlying 2-year old branch, as they were connected. In comparison, leaves which appear a new every year may gain most of their microbiome from the air. Therefore, investigation of endophyte community in leaves or green stem may show greater differences in the endophyte community between the two seasons. Another possible reason for the weak effect of season on endophyte community in this study is that it was not possible using the DGGE method to

determine the endophyte abundance. The endophytic community population size in the woody stem might therefore differ between seasons. The lack of a difference between seasons is supported by the results of this study that endophyte communities within apple woody stems are less affected by environment such as climate which are related to region, but more affected by soil condition and microclimate which are related to site. It also supported that any difference in the growth stage of the woody stem in the two seasons did not influence endophyte communities in this study.

Overall, tissue type, cultivar and site were the three main factors determining the structure of the endophyte communities in apple leaves and/or stems, with season having a lesser influence and region generally having no influence. This result supported the efficiency of the sampling strategy for endophyte isolation in Chapter 3 to obtain endophytes across a broad-spectrum for selecting biocontrol agents from leaf and stem of different apple cultivars across different sites. In this work enriched amplification of DNA from endophytes was also achieved by a combination of surface sterilisation to kill microorganisms on the tissue surface and then propidium monoazide (PMA) treatment for light dependent crosslinking of exposed DNA from non-viable surface located microorganisms, to inhibit their amplification by PCR (Nocker et al., 2007). Carini et al. (2017) reported that relic DNA led to inaccurate estimation of microbial diversity. Removing relic DNA before investigation of the relationship of microorganisms and orchard factors enabled more accurate interpretations of these communities to be carried out.

Since no sequencing of dominant DGGE bands to identify taxa was carried out in this study, whether some of the bands represent amplification of mitochondria or chloroplast DNA in plant samples is not known. However, Wicaksono (2016) found that since the bands from chloroplast DNA amplified using total bacterial primers were common to all tissue samples they had little effect on the comparison of community structure and richness of total bacteria between samples for evaluating the effect of factors such as site. In addition, the nested PCR approach which was used for total fungi, α-, β- and γ- proteobacteria and actinobacteria, allowed the analysis of minority populations present in apple tissues which were likely to contribute to differences in the community structure between samples.

The PCR-DGGE approach chosen to determine the community structure and richness of endophytes affected by different orchard factors was crucial to this study, as it allowed the complete endophyte communities (culturable and unculturable) to be evaluated in a cost effective way. Despite the possibility of some bands co-migrating, DGGE has been shown to allow nearly 100% of all possible sequence variations to be detected by incorporating GC-rich

sequences into one of the primers (Muyzer et al., 1993; Myers et al., 1985; Sheffield et al., 1989). The limitation of DGGE is that relative taxonomic abundance was not available as compared with next generation sequencing. Therefore, the core and rare endophytes related to orchard factors were unknown, however, DGGE can provide an overview of microbial communities.

This study provided new information on the complete endophyte community in apple tissues affected by different factors. This information will be useful in the development of sampling strategies for future research work investigating the complete or culturable endophyte communities. However, the selection of potential biocontrol agents for protecting against European canker still depends on the isolation of culturable endophytes from apple tissues. Actinobacteria, γ-proteobacteria, and total fungi were targeted for isolation and testing of biocontrol activity in Chapter 3, especially as *Pseudomonas* spp. belonging to γ-proteobacteria, actinobacteria and fungal endophytes are reported to be important sources of biocontrol candidates.

Chapter 3 Identification of *in vitro* biocontrol activity of endophytic bacteria and fungi isolated from apples leaves and stems

3.1 Introduction

Endophytic bacteria and fungi are microorganisms residing in living plant tissues without causing visible harm to the plant (Hallmann et al., 1997). Culturable endophytes can be isolated from surface-sterilised plant tissues (Miller et al., 2012; Trivedi et al., 2011). Some endophytes have beneficial effects on plant growth such as promoting growth and increasing resistance to diseases and abiotic stress (Bae et al., 2011; Porras-Alfaro & Bayman, 2011; Rajkumar et al., 2009; Varma et al., 1999). They are increasingly being targeted as a source of potential biological control agents (Backman & Sikora, 2008; Eljounaidi et al., 2016; Lodewyckx et al., 2002; Ryan et al., 2008). Research has been done to investigate the potential use of beneficial endophytes as biocontrol agents for controlling different plant diseases. de Almeida Lopes et al. (2018) found a strain of *Bacillus* sp. and strains of *Burkholderia* spp. had significant inhibition effect on *in vitro* growth of fungal pathogens such as *Sclerotinia sclerotiorum*, *Phomopsis sojae* and *Rhizoctonia solani* by producing antimicrobial compounds. Culturable bacterial endophytes with biocontrol potential were also reported by many other studies (Ren et al., 2013; Wicaksono et al., 2018; Wicaksono et al., 2017). Biocontrol potential of fungal endophytes isolated from plants have also been reported. For example, Lahlali and Hijri (2010) showed the endophytic fungi *Epicoccum nigrum* and *Trichoderma atroviride* to reduce infection of potato plants by *Rhizoctonia solani*. Bae et al. (2011) identified endophytic *Trichoderma* isolates having biocontrol activity against *Phytophthora capsici* in hot pepper (*Capsicum annuum*).

European canker, caused by the fungus *Neonectria ditissima,* is a severe trunk disease in apple orchards in warm and humid areas of New Zealand such as Waikato and Nelson. Disease incidence is lower in Hawke's Bay than in Nelson, because of the relatively dry climate in Hawke's Bay (Anon., 2013). In this study, apple shoots were collected both from orchards in Hawke's Bay and Nelson to compare the difference in the recovery of culturable endophytes antagonistic to *N. ditissima* in these two regions. Culturable endophytes isolated from apple shoots showing *in vitro* biocontrol activity against *N. ditissima* are promising candidates to be developed as biocontrol agents against European canker in apple orchards.

Beneficial endophytic bacteria and fungi can result in disease suppression through a range of different biocontrol mechanisms, including the production of siderophores with high iron affinities (Castignetti & Smarrelli, 1986), secretion of antibiotics (Fenton et al., 1992), production

of enzymes (Vijayalakshmi et al., 2016), production of secondary metabolites (Schulz et al., 1999), enhancing plant competition for nutrients and/or occupation in the plant (Gao et al., 2010) and inducing the systemic resistance of the host plant (Vu et al., 2006). In addition, beneficial endophytic fungi can inhibit the growth of pathogens by parasitism (Samuels et al., 2000). In this study, *in vitro* biocontrol assays for both endophytic bacteria and fungi focused on identifying antagonistic activity using dual plate assays, followed by culture filtrate, volatile and siderophore production assays to determine the role of antimicrobial compounds. Further, the detection of antibiotics encoding genes were determined for the bacterial endophytic isolates.

The objectives of this chapter were (i) to detect and characterise biocontrol activity of endophytic bacterial and fungal isolates obtained from apple leaves and stems against *N. ditissima*, (ii) to determine the general mechanisms of the selected antagonistic endophytic bacterial and fungal isolates, and (iii) to determine the potential of the selected antagonistic endophytic bacterial and fungal isolates to be developed as biocontrol agents for controlling European canker.

3.2 Materials and methods

3.2.1 Sampling strategies for endophyte isolation

Plant tissues (leaves and stems) from mature (over 5-year old) apple trees were collected in four orchard samplings and endophytes were isolated from these materials. All the tissue samples were surface-sterilised prior to isolating endophytes (Section 2.2.2).

3.2.1.1 Lincoln University Research Orchard (LU)

'Royal Gala' and 'Braeburn' tissue samples were collected from healthy trees without any obvious foliar or trunk disease symptoms in the Lincoln University Research Orchard (LU) in March 2015. The LU sampling was a preliminary experiment used to develop and refine the methodology for the main sampling. Two, one-year-old shoots with leaves and stems were collected, with each shoot from opposite sides of each of three 'Royal Gala' and three 'Braeburn' trees.

For each shoot sampled, five leaves were detached from the tip, middle and base of each shoot. For each shoot collected from each of two 'Royal Gala' trees, a stem segment from the tip of the shoot (approx. 5 cm long) and a stem segment from the base of the shoot (approx. 5 cm long) were also subsampled. The leaves and the basal and tip stem segments were used to test the endophyte isolation method.

3.2.1.2 Heritage varieties from Plant & Food Research Orchard in Hawke's Bay (HBHV)

To assess the diversity of culturable endophytes in the shoots of many apple varieties, 2-year old shoots were sampled from 35 heritage varieties and four commercial varieties ('Royal Gala', 'Braeburn', 'Gala' and 'Royal Gala Ten Hove') from the Plant & Food Research Orchard in Havelock North, Hawke's Bay (HBHV) in September 2015 (Appendix A3.1). The two trees per variety planted at the site were sampled by taking two shoots (2-year old) from the opposite sides of each tree. Surface-sterilised woody stem sections (approx. 7 cm long) taken from the base of the 2-year old shoot (not directly connected to the 3-year old shoot) were used for endophyte isolation.

3.2.1.3 Main sampling in spring (M1) and autumn (M2) for commercial varieties

The second and third leaves, green stem and woody stem portions from each shoot (Chapter 2: Figure 2.1) from the main samplings conducted in spring (November-December 2015) and autumn (April 2016) for the commercial varieties 'Royal Gala', 'Braeburn', 'Scifresh' and 'Scilate' from commercial orchards in Nelson (spring and autumn) and Hawke's Bay (spring only) (Chapter 2: Section 2.2.1) were used for endophyte isolation. There were a total of 29 orchards blocks for the M1 sampling and six of the 29 orchard blocks for the M2 sampling (Appendix A3.2), with six trees per block sampled.

3.2.2 Endophyte isolation and culture collection

3.2.2.1 Lincoln University Research Orchard (LU)

Isolation

Surface sterilised (Section 2.2.2) leaves, green stems and woody stems were cut into pieces (approx. 1 mm thick for stems) and five pieces plated onto each of triplicate plates of R2A (Difco, Becton, Dickinson and Company) plates for isolation of endophytic bacteria and onto each of triplicate plates of synthetic low-nutrient agar (SNA) (Appendix A3.3) (Berg et al., 2005) for endophytic fungi isolation. For each of the two 'Royal Gala' trees where both leaf and stem tissues for two shoots per tree were used for endophyte isolation, there was a total of 150 leaf pieces, 30 green stem pieces and 30 woody stem pieces per tree plated onto both R2A and SNA. For the third 'Royal Gala' tree and the three 'Braeburn' trees (two shoots per tree), there were 150 leaf pieces per tree plated on both R2A and SNA. The plates were incubated at 25°C in continuous darkness for four weeks, and the plates observed weekly for bacterial or fungal colonies growing from the tissue pieces.

Culture collection

All the bacterial isolates growing from the tissues on R2A plates were subcultured onto Nutrient Agar (NA; Oxoid, UK) after four weeks' incubation. As fungi can be distinguished based on their colony morphology, representative fungal isolates were selected based on their colony morphology on SNA plates. All the selected fungal isolates were subcultured onto potato dextrose agar (PDA; Difco, Becton, Dickinson and Company, USA) and grouped based on their colony morphology on PDA. In addition, some bacteria showing strong growth on the SNA agar plates and some fungi growing on R2A with diverse morphology were also subcultured as these may have represented different species with different nutrient requirements.

3.2.2.2 Heritage varieties from Plant & Food Research Orchard in Hawke's Bay (HBHV)

Isolation

Isolations from surface sterilised woody stem segments were carried out as described for the LU orchard isolations and plated onto triplicate plates of R2A and SNA (30 pieces per tree). Woody stem samples from 20 heritage varieties were plated with the bark attached, and the woody stems from the remaining 15 heritage varieties and the four commercial varieties were plated with the bark removed (Appendix A3.1). This is because early observations showed that some dominant fungi grew rapidly from the bark, inhibiting the isolation of other slow-growing fungal trunk endophytes. The plates were incubated at 25°C in continuous darkness for four weeks, with the plates observed weekly for bacterial or fungal colonies growing from the tissue pieces.

Culture collection

Ten bacterial isolates were randomly selected from each of the 35 heritage varieties using a random number selection method (https://www.randomizer.org/) and then subcultured onto NA. In addition, approximately 10 representative fungi with diverse colony morphologies on SNA were selected from each of the 35 heritage varieties and four commercial varieties and subcultured onto PDA.

3.2.2.3 Main sampling in spring (M1) and autumn (M2) for commercial varieties

Isolation

Surface sterilised leaves (the second and third leaves), green and woody stem portions were used for isolation of endophytic bacteria and fungi using the same methods as described for the previous two samplings. In addition, isolation of actinobacteria was attempted from the tissue samples taken in the spring sampling, by plating five pieces per tissue onto an Actinomycete Isolation Agar (AIA; Difco, Becton, Dickinson and Company, USA) plate. Only one plate was set up

for each tissue sample. Since only one actinobacterial strain was isolated in the spring sample there was no attempt to isolate actinobacteria from the autumn tissue samples. The plates were incubated at 25°C in continuous darkness for four weeks, with the plates observed weekly for bacterial, actinobacterial and fungal colonies growing from the tissue pieces.

Culture collection

From each R2A plate, the bacterial isolates growing from two of the five tissue pieces (40%), which were randomly selected using a dice, were subcultured onto NA (first random selection of bacteria). From these cultures, 25 bacterial isolates (M1)/10 bacterial isolates (M2) were randomly selected per cultivar per block (second random selection of bacteria using the random number selection method) and then subculture onto NA. For endophytic fungi, 25 fungal isolates (M1)/10 fungal isolates (M2) were selected per cultivar per block, based on diverse colony morphology onto SNA as the criterion, and then subcultured onto PDA. Therefore, the aim was to obtain 725 bacterial isolates and 725 fungal isolates from the M1 sampling (29 blocks) and 60 bacterial isolates and 60 fungal isolates from the M2 sampling (6 blocks).

3.2.2.4 Bacterial and fungal culture storage

All endophytic bacteria were stored in a final concentration of 20% glycerol (LabServ, Thermo Fisher Scientific, New Zealand) at -80°C. A representative number of endophytic fungi were stored in a preservation solution with a final concentration of 60% glycerol, 10% glucose, 10% peptone (pancreatic casein) and 1% yeast extract at -80°C (Dr Wisnu Adi Wicaksono Pers. Comm.).

3.2.3 Identification of endophytic fungal isolates by colony morphology on PDA

All fungal cultures were purified by subculturing for twice after isolation, and then were grouped based on their colony morphology on PDA. Specific terminology was used to describe common colony types of fungi (http://www.microbiologyonline.org.uk/teachers/observing-microbes/observing-fungi-in-a-petri-dish). These were pigmentation (e.g. white, buff, red or purple), form (e.g. circular, irregular, filamentous or rhizoid), elevation (e.g. raised, convex, flat, umbonate or crateriform), margin (e.g. entire, undulate, filiform, curled or lobate), surface (e.g. smooth or wrinkled) and opacity (e.g. transparent or opaque).

Representatives of the dominant morphology groups were identified by sequencing of the ribosomal internal transcribed spacer (ITS) region amplified using the primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Guillamón et al., 1998). Fungal mycelium of each isolate was picked up by a sterile 0.1-10 µL pipette tip for extracting DNA, followed by PCR amplification using the Extract-N-Amp™ Plant PCR Kits (Sigma-Aldrich,

USA) following the manufacturer's instructions. PCR amplification was performed in a thermal cycler (Applied Biosystem Veriti, Thermo fisher Scientific, USA) in a total volume of 20 µL containing 4 µL ultra-pure water (Life Technologies, Thermo Fisher Scientific, USA), 10 µL Extract-N-Amp PCR ReadyMix, 1 µL of of each forward and reverse primer (0.25 µM) (Integrated DNA Technologies, Australia) and 4 µL DNA extract. The PCR conditions were as follows: an initial denaturation at 94°C for 2 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, with final extension at 72°C for 7 min. PCR products along with the 1kb plus DNA ladder (Invitrogen, Life Technologies, Thermo Fisher Scientific, USA) were visualised by 1% agarose gel electrophoresis. A 5 µL aliquot of each PCR product was mixed with 1 µL of loading dye and loaded onto a 1% agarose gel (Bioline, USA) in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM NaEDTA; pH 8.0) and separated by electrophoresis for 1 h at 100 V. Gels were stained in ethidium bromide solution (0.5 µg/mL, 0.5 × TAE) for 15 min, destained with water then visualized under ultra-violet light using a UVIreader (UVItec, UK). Approximately 600 bp of the ITS region was expected to be amplified. The PCR products were sequenced directly using primer ITS4 at the Lincoln University Sequencing Facility. The sequences obtained were viewed and trimmed using FinchTV (http://jblseqdat.bioc.cam.ac.uk/gnmweb/download/soft/FinchTV_1.4/doc/) to remove any ambiguous regions. The sequences were then identified by comparing with those of known origin using the Basic Local Alignment Search Tool (BLAST) in the GenBank nucleotide database (NCBI; https://www.ncbi.nlm.nih.gov).

3.2.4 Culturable fungi community structure analysis in different apple varieties from the HBHV sampling and in different apple blocks from the M1 and M2 sampling

Each fungal morphotype (Section 3.2.3) was recorded as present (1) or absent (0) per variety for the HBHV sampling (with 35 heritage varieties and four commercial varieties) and per block for the M1 and M2 sampling (23 blocks for 'Royal Gala' and 'Braeburn', with six blocks of which sampled in both of the samplings; Appendix A2.1).

Apple shoots in the HBHV sampling were collected from the same site (Plant & Food Research Orchard in Hawke's Bay) and the effect of apple variety on the endophytic culturable fungi community was analysed in this site. Apple shoots in the M1 and M2 samplings were collected from different regions (Nelson/Hawke's Bay), sites, varieties, management practices (IFP/organic), seasons (spring for the M1 sampling and autumn for the M2 sampling) and sites with both low (≤ 30%) and high (≥ 50%) European canker infection level blocks. Firstly, effects of these factors on the cultured fungal community were analysed for all 23 blocks including the six blocks sampled in both seasons. Then, selected orchard blocks were used to show the effect of

each factor including region, variety, season and infection level on the culturable fungal community. Effects of region was analysed for sites 1-8, with both 'Royal Gala' and 'Braeburn', in which sites 7 and 8 were organic orchards. Effect of variety was analysed using blocks in three test sites (sites 2, 5 and 6) with 'Royal Gala', 'Braeburn' and 'Scifresh'. Effect of season was analysed using the blocks sampled in spring (M1 sampling) and autumn (M2 sampling) in three test sites (sites 1, 2 and 4) with 'Royal Gala' and 'Braeburn'. Effect of infection level of the block was analysed using the blocks in three test sites (sites 2, 5 and 9) with high and low infection blocks of 'Royal Gala' in site 2, 'Scifresh' in site 5 and 'Braeburn' in site 9.

Cluster analysis with SIMPROF test, based on Jaccard similarity and distance/dissimilarity (Clarke & Warwick, 2001), was performed to identify the difference in the culturable fungi community (presence and absence of a fungal group) affected by varieties in the HBHV sampling and affected by the factors of region, site, variety, management practice, season and infection level of the block in the M1 and M2 samplings. PERMANOVA test was performed to determine the significant differences in the similarity distance in the culturable fungi communities between the clusters. For the M1 and M2 samplings, an ANOSIM (analysis of similarities) one-way test was performed to determine the significant differences in culturable fungi communities in apple shoots collected from different blocks affected by the different factors including region, site, variety, management practice, season and infection level. Finally, a SIMPER (similarity percentages) analysis based on Bray-Curtis similarity was used to determine the contribution of culturable fungal groups to the difference between clusters for the HBHV sampling and to the difference between the spring and autumn samplings for the M1 and M2 samplings. All the analyses were performed in Primer 7 statistical package (Primer-E, Plymouth Marine Laboratory, UK). All null-hypothesis significance tests were performed with $\alpha = 0.05$.

3.2.5 Dual culture plate assays for culturable endophytic bacteria and fungi against *Neonectria ditissima* ICMP14417

3.2.5.1 Endophytic bacteria

A primary dual culture assay was conducted to analyse the antagonistic activity of each of the endophytic bacterium collected from LU (n = 87), HBHV (n = 134), M1 (n = 725) and M2 (n = 58) against *N. ditissima* isolate ICMP14417. Endophytic bacteria derived from the LU sampling were used in a pilot study for the primary dual culture plate assay. In the primary dual culture assay for endophytic bacteria, a 5 mm diameter mycelial disc was taken from the margin of a 2-3-week-old colony of *N. ditissima* ICMP14417 grown on PDA and placed in the centre of a Petri dish containing Waksman agar (Appendix A3.3) (Berg et al., 2002). Then, each plate was inoculated

with four endophytic bacteria using sterilised toothpicks, with each dotted at one of four equidistant points around the *N. ditissima* colonised agar disc (1 cm away from the edge of the plate). One of the endophytic bacteria was a positive control, isolate W4R11 (*Burkholderia* sp.) (Wicaksono et al., 2016), which had been shown as being antagonistic to *N. ditissima* ICMP14417. The other three were bacterial isolates from the current study. Waksman agar plates inoculated centrally with *N. ditissima* ICMP14417 only, served as negative controls. Triplicate plates were set up and incubated at 20°C in 12 h dark/12 h light.

The interaction between the endophytic bacteria and *N. ditissima* ICMP14417 was observed 12 days after inoculation for the bacteria isolated from the LU sampling (pilot study) and 17 days after inoculation for all other samplings (HBHV, M1 and M2 samplings). The interactions types between the endophytic bacteria and *N. ditissima* ICMP14417 were classified into six types, outlined as follows:

Type A- no inhibitory effects, where the endophytic bacterial isolate is almost totally overgrown by *N. ditissima*, **Type B**- *N. ditissima* colony is not reduced in size, contacts with the bacterial colony and slight overgrowth of the bacterial colony by *N. ditissima*, **Type C**- *N. ditissima* colony is not reduced in size, contacts with the bacterial colony, but without overgrowth of the bacterial colony by *N. ditissima*, **Type D**- *N. ditissima* colony is reduced in size, contacts with the bacterial colony and with slight overgrowth of the bacterial colony by *N. ditissima*, **Type E**- *N. ditissima* colony is reduced in size, and a barrage line but no clear inhibition zone seen for *N. ditissima*, zone, no overgrowth of the bacterial colony by *N. ditissima*, with/without dark colouration of the *N. ditissima* colony, and **Type F**- *N. ditissima* colony is reduced in size and a clear inhibition zone seen for *N. ditissima*, with/without barrage line or dark colouration of the *N. ditissima* colony. Interaction types D, E and F are considered to be antagonistic and selected for the secondary dual culture assay.

In the secondary dual culture assay, one endophytic bacterium was dotted at four equidistant points around the *N. ditissima* colonised agar disc (1 cm from the edge of the plate). Triplicate plates were set up and plates were incubated at 20°C under 12 h dark/12 h light for 17 days. In this assay, radial growth of *N. ditissima* ICMP14417 colonies on the dual culture plates towards the bacterial isolate colonies were measured. Results were expressed as means of the *N. ditissima* ICMP14417 colony radius (mm) and used to calculate the percent inhibition of *N. ditissima* ICMP14417 by the endophytic bacterial isolates as described by Zhang et al. (2015):

$$= \left(\frac{\text{Radius } N.ditissima \text{ colony in negative control} - \text{Radius } N.ditissima \text{ colony in treatment}}{\text{Radius } N.ditissima \text{ colony in negative control}} \right) \times 100$$

3.2.5.2 Endophytic fungi

Primary dual culture assay

Eighty-seven endophytic fungal isolates from the M1 sampling were randomly selected from each fungal morphotype using a dice (representing the 33 fungal groups based on colony morphology), with no more than 10 isolates for each morphotype (uneven selection). The 87 representative fungal isolates were tested for their *in vitro* antagonistic activity against *N. ditissima* ICMP14417 in a primary dual culture assay.

In the primary dual culture assay, a 5 mm diameter mycelial disc of *N. ditissima* ICMP14417 from a 2-week-old colony grown on PDA was placed on one side of a Petri dish containing PDA (1 cm away from the edge). A 5 mm diameter mycelial disc of each endophytic fungus (5-30-day-old depending on the growth rate of each fungus) grown on PDA was placed on the opposite side of the Petri dish (also 1 cm from the edge of the plate), one pairing per plate. PDA plates inoculated only with *N. ditissima* ICMP14417 served as negative controls. Triplicate plates were set up and plates were incubated at 20°C in 12 h dark/12 h light for 22 days. The interaction types between the endophytic fungi and *N. ditissima* ICMP14417 were observed 22 days after inoculation. The interactions were classified into four types which are outlined as follows: The **Type A**- fungal endophyte is overgrown by *N. ditissima*. **Type B**- the hyphae of the two organisms approach one another and stop growing (some isolates show dark and dense line). **Type C**- the growing margins of the two colonies meet, *N. ditissima* is inhibited and overgrown by the fungal endophyte. **Type D**- the growth of *N. ditissima* is inhibited at a distance leaving a clear zone of inhibition between the two organisms. Type C and D interactions are considered to be antagonistic and selected for the secondary dual culture assay.

Secondary dual culture assay

In the secondary dual culture assay, to account for the differences in relative growth rates and to minimise this effect on the inhibition results the fungal isolates selected from the primary assay were divided into three groups based on their growth rate on PDA. These being fast-growing (F), medium-growing (M) and slow-growing (S) groups. The fungal isolates belonging to the fast-growing group and the slow-growing group were inoculated 10 days after and 10 days before the inoculation of *N. ditissima* ICMP14417, respectively. The medium-growing fungal isolates were inoculated simultaneously with *N. ditissima* ICMP14417. PDA plates inoculated only with *N. ditissima* ICMP14417 served as the negative control. Triplicate plates were set up and plates were incubated at 20°C under 12 h dark/12 h light. The interaction between the endophytic fungi and

N. ditissima ICMP14417 were observed 22 days after inoculation with *N. ditissima* ICMP14417 based on the same interactions types as outlined for the primary dual culture assay.

3.2.6 Identification and phylogenetic analysis of the selected culturable endophytic bacteria and fungi by DNA sequencing

Endophytic bacterial and fungal isolates showing antagonistic activity against *N. ditissima* ICMP14417 in the dual culture plating assays were identified using the sequence of the 16S rRNA and ITS region, respectively. DNA of fungal isolates was extracted and amplified by PCR for the ITS region using the Extract-N-Amp™ Plant PCR Kits as described in the Section 3.2.3. DNA of bacterial isolates was extracted using the rapid Extract-N-Amp™ Plant PCR Kits following the manufacturer's instructions. For the bacterial isolates 21-606(28)b, 26-771(6)b and 26-771(23)b which were not successfully extracted and amplified using the Extract-N-Amp™ Plant PCR Kit, DNA were extracted using the UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, USA) and then amplified using the FastStart Taq DNA Polymerase, dNTPack kit (Roche, Germany) following the manufacturer's instructions. Approximately 1,500 bp of the 16S rRNA gene was amplified using the primers F27 (5'-AGA GTT TGA TCM TGG CTC AG-3') and R1494 (5'-CTA CGG YTA CCT TGT TAC GAC-3') for bacteria identification (Neilan et al., 1997). In addition, the reverse primer 518R (5'- ATT ACC GCG GCT GCT GG-3') was used in place of R1494 for the bacterial isolates from which the sequence obtained from F27 and R1494 could not be assembled (Wicaksono et al., 2016). Amplification of the 16S rRNA gene of bacteria using the Extract-N-Amp™ Plant PCR Kits was conducted as described for fungal isolates in Section 3.2.3. PCR amplification of the 16S rRNA gene for the bacteria using the FastStart Taq DNA Polymerase, dNTPack kit was performed in a total volume of 25 µL containing 18.75 µL ultra-pure water, 2.5 µL 10 × PCR buffer with 20 mM $MgCl_2$ (a final concentration of 2 mM $MgCl_2$), 0.2 mM dNTPs, 0.2 µM of each forward and reverse primers, 1.25 U FastStart *Taq* DNA Polymerase and 1 µL DNA template. PCR conditions for bacterial isolates were the same as described for fungal isolates in the Section 3.2.3.

PCR products were visualised by 1% agarose gel electrophoresis (Section 3.2.3) and sequenced directly at the Lincoln University Sequencing Facility. The sequences obtained were viewed and trimmed using FinchTV to remove any ambiguous regions. The two sequences derived from a single PCR product using the forward and reverse primer, respectively, were assembled using DNAMAN 4.0 (Lynnon Biosoft, Canada) only if there was an overlap region between the two sequences. The resulting sequences were identified by comparison to those of known origin using the Basic Local Alignment Search Tool (BLAST) in the GenBank nucleotide database (NCBI;

https://www.ncbi.nlm.nih.gov). The sequences were aligned and the distance matrices and phylogenetic trees were calculated by unweighted pair group algorithm with arithmetic average (UPGMA) in Geneious R10 software (Biomatters, New Zealand).

Any fungal or bacterial isolates identified as potential plant pathogens based on their identification were excluded from the remaining studies.

3.2.7 Dual culture plating assays of selected endophytic bacteria and fungi against three *Neonectria ditissima* strains

All endophytic bacterial that inhibited *N. ditissima* ICMP14417 and that were not potential pathogens were used in further work to determine their *in vitro* biocontrol activity against two further *N. ditissima* isolates, MW15c1 and RS324p, obtained from Drs Monika Walter (Plant & Food Research, Motueka) and Reiny Scheper (Plant & Food Research, Hawke's Bay) as described in Section 3.2.5.

To assess the endophytic bacteria activity, the radius of each *N. ditissima* isolate colony was measured 17 days after inoculation and the percent inhibition was calculated as described in Section 3.2.5.1. The radial growth of *N. ditissima* was compared within the treatment (inhibition effect of endophytic bacterial isolates on each *N. ditissima* isolate) and among the treatments (inhibition effect of endophytic bacterial isolates across the three *N. ditissima* isolates). The overall effect of inoculation with endophytic bacteria on the radial growth of the three *N. ditissima* isolates was analysed with nonparametric Friedman two-way ANOVA in SPSS Statistics 24 (IBM, USA). The effect of endophytic bacteria on the radial growth of each *N. ditissima* isolate was analysed using Kruskal-Wallis one-way ANOVA test, followed by pairwise comparisons performed in SPSS Statistics 24.

The following functional assays were focused on the selected endophytic bacteria. Therefore, only 11 of the 15 selected endophytic fungi were tested for their interaction with the three *N. ditissima* strains and observed 22 days after inoculation to determine the type of interaction as described in Section 3.2.5.2.

3.2.8 Effect of pathogen presence on production of inhibitory compounds by selected endophytic bacteria

Antagonistic bacteria inhibit the growth of pathogens by production of inhibitory compounds. The dual plating assay was repeated to determine whether the inhibitory compounds were secreted in the absence of the pathogen, *N. ditissima* ICMP14417 (constitutively secreted) or induced by the presence of the pathogen. Each of the endophytic bacteria were inoculated 7, 4 and 1 day (s)

prior to *N. ditissima* ICMP14417 inoculation using the same dual culture method described in Section 3.2.4.1. If there was no difference in the radial growth of the *N. ditissima* colony in the three treatments, production of inhibitory compounds by the tested endophytic bacterial isolate was considered to be induced by *N. ditissima*. In contrast, a difference in the colony radial growth indicated that the production of inhibitory compounds by the tested endophytic bacterial isolate occurred in the absence of *N. ditissima*. Radial growth of *N. ditissima* ICMP14417 dual-cultured with each tested bacterial isolate was measured 17 days after inoculation of *N. ditissima* in the direction of the bacterial isolate colony. Radial growth of *N. ditissima* ICMP14417 was analysed by one-way ANOVA followed by Fisher's protected LSD test at $p \leq 0.05$ using Minitab 17 (Lead Technologies, Australia).

This experiment was not conducted for the selected endophytic fungal isolates, as there was a high variation in the growth rate of the different fungal isolates and the radial growth of *N. ditissima* was not able to be measured accurately.

3.2.9 Cell-free filtrate culture assays for the selected endophytic bacteria and fungi

A single colony of each selected bacterial isolate (2-day-old NA culture) was inoculated into 1 mL of nutrient broth (NB; Difco, Becton, Dickinson and Company, USA) and incubated for 8 h at 28°C on an orbital shaker at 200 rpm. Then, 100 µL culture of each bacterium was inoculated into 120 mL Waksman broth (Appendix A3.3) (Berg et al., 2002) and then incubated on an orbital shaker at 20°C at around 120 rpm. Fifty millilitres of bacterial culture were collected after 16 h and 38 h respectively. Each bacterial culture was centrifuged for 15 min at 12,100 x *g* to pellet the bacterial cells. The cell-free (CF) culture supernatant was filtered through a sterile 0.22 µm pore biological membrane filter (Rotilabo Spritzenfilter, Carl Roth, Germany) under sterile conditions (Zhang et al., 2015). Cell-free culture filtrate obtained after 16 h and 38 h was incorporated into autoclaved Waksman agar cooled to 50°C to obtain 1%, 10% and 30% (v/v) filtrate incorporated agar and used to determine the effect on *N. ditissima* ICMP14417 growth. Three plates for each filtrate concentration per bacterial isolate treatment were inoculated centrally with a 5 mm disc taken from the margin of a *N. ditissima* isolate ICMP14417 colony growing on PDA. Controls consisted of *N. ditissima* ICMP14417 inoculated on unamended Waksman agar.The radial growth of *N. ditissima* ICMP14417 was measured after 14 days growth at 20°C under 12 h dark/12 h light. The radial growth of *N. ditissima* ICMP14417 in each treatment was compared with the negative control using a one-way Kruskal-Wallis test with pairwise comparison performed in the SPSS statistics 24.

Only nine of the 15 selected endophytic fungi were tested in this assay. They were three fast-

growing fungi (1-38f, 2-51f and 20-578f), three medium-growing isolates (34-1026(3)f, 34-1029(1)f and 35-1049(2)f) and three slow-growing isolates (3-73f, 36-107Xf and 36-1072f). Five mycelial discs (5 mm) of the fast-growing endophytic isolates (5-day-old PDA cultures), medium-growing endophytic fungi (15-day-old PDA cultures) and slow-growing endophytic fungi (25-day-old PDA cultures) were inoculated into 100 mL potato dextrose broth (PDB; Difco™, Becton, Dickinson and Company, USA) in 250 mL flask, respectively. After incubation on an orbital shaker at around 100 rpm (Raut et al., 2014) at room temperature the fungal broth culture was harvested after 5 days for fast-growing fungi, after 15 days for medium-growing fungi and after 30 days for slow-growing fungi. The liquid culture of each fungus was filtered through sterilised Miracloth (Calbiochem, EMD Millipore, USA) to remover the mycelia, followed by centrifugation for 15 min at 12,100 xg to remove any remaining fungal mycelium and spores. The supernatant was filtered through a sterile 0.45 µm pore biological membrane filter (Rotilabo spritzenfilter, Carl Roth, Germany) under sterile conditions to obtain CF culture filtrate of each fungus. Cell-free culture filtrate for each fungus was then incorporated into sterile PDA cooled to 50°C to obtain a final concentration of 1% and 10%. As described for the bacterial CF culture filtrate amended agar, three plates per fungal isolate treatment were inoculated centrally with a 5 mm *N. ditissima* isolate ICMP14417 colonised agar disc. Controls consisted of *N. ditissima* inoculated on unamended PDA. After 22 days incubation at 20°C under 12 h dark/12 h light, the radial growth of *N. ditissima* ICMP14417 was measured and compared in each treatment using a one-way ANOVA using Minitab 17 (Lead Technologies, Australia) and significant difference between means determined using Fisher's protected LSD test at $p \leq 0.05$.

3.2.10 Siderophore production assays for the selected endophytic bacteria and fungi

Chrome Azurol S (CAS) assay was used to test the ability of the selected bacteria and fungi to produce siderophores in a solid medium. CAS agar was prepared according to the method described by Louden et al. (2011). A single colony of each endophytic bacterium (2-day-old) was inoculated into 1 mL NB and incubated in an orbital shaker at 28°C and 200 rpm overnight. Each of three replicate CAS agar plates was inoculated with three different test isolates and one isolate (unidentified) known to be positive for siderophore production on each replicate plate. Plates were observed after three days' growth for production of an orange halo, indicating siderophore production, around the endophytic bacterial colonies. The CAS reaction was determined by measuring the radius of the orange halo produced by the bacterial isolates three days after inoculation.

In addition, siderophore production for all the selected endophytic bacteria and fungi were assessed using the modified CAS assay method described by Milagres et al. (1999). For endophytic bacterial isolates, Petri dishes containing NA served as the culture medium. Once the agar had set, one half of the NA in the Petri dish was removed and replaced with CAS-blue agar. The NA half was inoculated with 10 µL of the overnight liquid culture of one of the bacterial isolates, as close as possible to the middle point of the borderline between the two media. Uninoculated plates served as negative controls. Three replicate plates were set up for each tested bacterial isolate and all plates incubated at 25°C in darkness. Strain growth rates were monitored daily and expressed as the number of days required by the bacterial colonies to reach but not cross the borderline between the two agars.

For endophytic fungal isolates, PDA was used as the culture medium. The halves containing PDA were inoculated with a 5 mm mycelia disc of one of the fungal isolates (10 mm from the edge of the plate). Uninoculated plates served as negative controls. Three replicate plates were set up for each tested fungal isolate and all were incubated at 25°C in darkness. The growth rates of the isolates were monitored daily and expressed as the number of days required by the fungal mycelia to cover the halves of the Petri plates containing PDA. The CAS reaction of each tested bacterial and fungal isolate was determined by measuring the advancement of the colour-change front in the CAS-blue agar, starting from the borderline between the two media (in mm). The colour change on the CAS-blue agar half was recorded. CAS reaction rates were calculated from the distance of the advancing colour-change front for the CAS-blue agar relative to the incubation time (days) and expressed as mm per day. Based on the reaction rates, the interactions were classified into four groups: '-' 0.0 (no colour change); '±' 0.1- 0.2; '+' 0.3-1.0; '++' 1.1-2.0 and '+++' 2.0-4.0 mm/day.

3.2.11 Volatile assays for the selected endophytic bacteria and fungi

For testing the volatile compounds produced by the selected endophytic bacteria, sandwich plates were used by sealing a NA plate inoculated with one of the endophytic bacteria to a PDA plate inoculated with *N. ditissima* ICMP14417. The endophytic bacteria were streaked onto NA plates and 5 mm diameter *N. ditissima* ICMP14417 mycelial colonised agar discs were centrally inoculated onto PDA plates. The bacteria and the *N. ditissima* colony were in a common gaseous environment but were not in direct contact with each other. The negative control consisted of a *N. ditissima* ICMP14417 on a PDA plate being sealed to an uninoculated NA plate. Each assay was performed in triplicate. The sandwich plates were sealed tightly with two layers of parafilm (Breathe-Easy sealing membrane, Sigma) and one layer of masking tape to avoid the escape of the

vapour phase and incubated at 20°C for 17 days under 12 h dark/12 h light. Radial growth of *N. ditissima* ICMP14417 was measured.

For testing the selected endophytic fungi, split compartment Petri dishes (NEST, Nest Biotechnology, China) were used, with both sides of the Petri dish containing PDA. A 5 mm diameter mycelial colonised agar disc of one of the test fungi was inoculated in one half chamber of the plate (10 mm from the edge of the plate), and a 5 mm diameter mycelial disc of *N. ditissima* ICMP14417 was inoculated in the other half chamber of the plate (1 cm from the edge of the plate). *Neonectria ditissima* ICMP14417 was inoculated on the same day as the slow- and medium-growing endophytic fungi, and eight days prior to fast-growing endophytic fungi. The negative control consisted of only *N. ditissima* ICMP14417 in one half chamber of the PDA plate. Each treatment combination was performed in triplicate. All the plates were sealed tightly as described for the endophytic bacteria assay. All the plates were incubated at 20°C under 12 h dark/12 h light. Radial growth of *N. ditissima* ICMP14417 towards the test fungi was measured 14 days after the inoculation of *N. ditissima* ICMP14417 and the mean of radial growth was calculated based on the triplicated plates. The radial growth of *N. ditissima* ICMP14417 in each treatment was compared by one-way ANOVA using Minitab 17 followed by Fisher's LSD test.

3.2.12 Detection of antibiotic producing genes in the selected endophytic bacteria using PCR

In order to better understand the underlying mechanisms by which the selected antagonistic endophytic bacteria inhibited the growth of *N. ditissima*, genes encoding antibiotic production were detected in the selected endophytic bacteria using PCR. PCR was attempted for five genes encoding the production of the antibiotics 2, 4- diacetylphloroglucinol (*phlD* gene), phenazine (*phzC* gene), pyrrolnitrin (*prnC* gene), pyoluteorin (*pltC* gene), and hydrogen cyanide (*hcnBC* gene) using DNA from the isolates identified as *Pseudomonas* spp. in Section 3.2.6 (de Souza & Raaijmakers, 2003; Mavrodi et al., 2001; Mazurier et al., 2009; McSpadden Gardener et al., 2001; Ramette et al., 2003). For isolates identified as *Bacillus* spp. in Section 3.2.6, amplification of four genes, those encoding the production of the antibiotics surfactin (*sfp* gene), iturin A (*ItuD* gene), fengycin (*FenD* gene) and bacillomycin D (*BamC* gene) were attempted using PCR (Gond et al., 2015) (Table 3.1).

DNA from the endophytic bacteria was extracted using the PureGene kit (Qiagen) following the manufacturers' procedure. PCR were performed in a thermal cycler (Applied Biosystems Veriti, Thermo Fisher Scientific, USA) in a total volume of 25 µL reaction mixture containing the same concentrations of 10 × PCR buffer and dNTPs as they were described in Section 3.2.6, 0.4 µM of each forward and reverse primers (Integrated DNA Technologies, USA), 1 U FastStart *Taq* DNA

polymerase (Roche, Germany) and 1 µL DNA template. The primer sequences and PCR conditions used are shown in Table 3.1. PCR products were visualised by 1% agarose gel electrophoresis (Section 3.2.3).

In an attempt to increase the sensitivity of detection of the antibiotic encoding genes, the PCR conditions were modified as described in Appendix A3.4.

For bands of interest, the PCR product with the expected size for each antibiotic producing gene was excised and purified using the HighPure PCR product purification kit (Roche, Germany). The PCR-amplified antibiotic producing genes were sequenced directly and sequence results were compared with non-redundant protein sequences (nr) present in the NCBI database (www.ncbi.nlm.nih.gov) using the BLASTX algorithm.

Table 3.1 Details of the primers and PCR conditions used to amplify five different antibiotics producing genes for *Pseudomonas* spp. isolates and four antibiotics producing genes for *Bacillus* spp. isolates.

Species	Antibiotic	Gene target	Primer	Sequence (5'-3')	PCR conditions	Amplicon size (bp)	Reference
Pseudomonas spp.	2,4-diacetylphloroglucinol	*phlD*	B2BF	ACC CAC CGC AGC ATC GTT TAT GAG C	95°C for 3 min 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min	600	McSpadden Gardener et al. (2001)
			BPR4	CCG CCG GTA TGG AAG ATG AAA AAG TC	72°C for 5 min		
	phenazine	*phzC*	PHZJR1	CAG GGC CG(G/C) (A/G)(C/T)A TTT CTC GGT TCT	94°C for 2 min 30 cycles of 94°C for 1 min, 67°C for 45 s, 72°C for 1 min	522	Mazurier et al. (2009)
			PHZJR2	GCG CGG GTC GCA CAG G CT T TTG TA	72°C for 10 min		
	pyrrolnitrin	*prnC*	PrnCf	CCA CAA GCC CGG CCA GGA GC	94°C for 2 min 30 cycles of 94°C for 1 min, 58°C for 45 s, 72°C for 1 min	719	Mavrodi et al. (2001)
			PrnCr	GAG AAG AGC GGG TCG ATG AAG CC	72°C for 10 min		
	pyoluteorin	*pltC*	PLTC1	AAC AGA TCG CCC CGG TAC AGA ACG	95°C for 2 min 30 cycles of 95°C for 2 min, 67°C for 1min, 72°C for 1 min	438	de Souza and Raaijmakers (2003)
			PLTC2	AGG CCC GGA CAC TCA AGA AAC TCG	72°C for 10 min		
	hydrogen cyanide	*hcnBC*	ACa	ACT GCC AGG GGC GGA TGT GC	94°C for 2 min 30 s 30 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 1 min	549	Ramette et al. (2003)
			ACb	ACG ATG TGC TCG GCG TAC	72°C for 10 min		

Table 3.1 continued

Species	Antibiotic	Gene target	Primer	Sequence (5'-3')	PCR conditions	Amplicon size (bp)	Reference
Bacillus spp.	Surfactin	*sfp* gene	sfp-f	ATGAAGATTTACGGAATTTA	95°C for 5 min 30 cycles of 94°C for 1 min,	675	Gond et al. (2015)
			sfp-r	TTATAAAAGCTCTTCGTACG	55°C for 1 min, 72°C for 1 min 72°C for 10 min		
	Iturin A	*ItuD*	ItuD1f	GATGCGATCTCCTTGGATGT	95°C for 5 min 30 cycles of 94°C for 1 min,	647	Gond et al. (2015)
			ItuD1r	ATCGTCATGTGCTGCTTGAG	55°C for 1 min, 72°C for 1 min 72°C for 10 min		
	Fengycin	*FenD*	FenD1f	TTTGGCAGCAGGAGAAGTTT	95°C for 5 min 30 cycles of 94°C for 1 min, 58	964	Gond et al. (2015)
			FenD1r	GCTGTCCGTTCTGCTTTTTC	°C for 1 min, 72°C for 1 min 72°C for 10 min		
	Bacillomycin D	*Bam C*	Bacc1f	GAAGGACACGGAGAGAGTC	95°C for 5 min 30 cycles of 94°C for 1 min,	875	Gond et al. (2015)
			Bacc1r	CGCTGATGACTGTTCATGCT	55°C for 1 min, 72°C for 1 min 72°C for 10 min		

3.3 Results

3.3.1 Endophyte collection

No microbial growth was observed after 5 days of incubation at 25°C in the darkness when an aliquot of the final rinse water was plated on R2A and SNA indicating that the surface sterilisation protocol effectively inhibited the growth of bacteria and fungi from the phylloplane.

Pure cultures of endophytic bacteria (n = 1004) and fungi (n = 1296) were recovered from the four samplings (Table 3.2). Endophytic bacteria were only collected from the 35 heritage varieties, resulting in 350 bacterial isolates in total in the HBHV sampling. These were stored on NA plates at 7°C for around 6 months, which reduced viability with only 134 isolates retaining viability (Table 3.2). Only the viable bacterial isolates were subcultured further which resulted in uneven, non-random, selection from the plant tissue. In the HBHV sampling, 316 endophytic fungi were collected from the 35 heritage varieties and 25 isolates were collected from four commercial varieties ('Royal Gala', 'Braeburn', 'Gala' and 'Royal Gala Ten Hove'). Isolation of actinobacteria was only attempted from the M1 sampling and only one actinobacterial isolate was succesfully isolated which was identified based on colony morphology (Table 3.2). No further attempts to isolate actinobacteria from the remaining sampling was done.

Table 3.2 Number of endophytic bacterial, fungal and actinobacterial isolates recovered at each sampling.

Isolates	Number of isolates			
	LU	HBHV	M1	M2
Bacteria	87	134 [h]	725	58
Fungi	170	316[h]/ 25[c]	725	60
Actinobacteria	NA	NA	1	NA

NA- not applicable since actinobacteria isolations were not attempted, LU- Lincoln University research orchard, M1- Hawke's Bay and Nelson sampling in spring 2015, M2- Nelson sampling in autumn 2016, and HBHV- heritage varieties sampling in the Plant & Food Research orchard in Hawke's Bay. [h] fungal isolates were from the 35 heritage varieties, [c] fungal isolates were from the four commercial varieties which were also sampled in the HBHV sampling.

Fungal isolates collected from LU, HBHV, M1 and M2 samplings were grouped based on their colony morphology on PDA. A total of 39 morphology groups were observed (Appendix A3.5), with 6 groups in the LU sampling, 24 groups in the HBHV sampling, 33 groups in the M1 sampling and 15 groups in the M2 sampling (Table 3.3; Figure 3.1). A representative number of fungal isolates (n =

42 from LU, n = 134 from HBHV, n = 87 from M1 and n = 69 from M2) representing the 39 morphology groups were stored at -80°C.

The number of isolates in the collection did not reveal the true abundance of each fungal morphotype, because the number of isolates for the different morphotypes were not selected evenly per variety. However, it demonstrated the presence and absence of each culturable fungal morphotype in different samplings and selection frequency of them. The three most abundant morphotypes Gf5, Gf11 and Gf20 with high selection frequency in the M1 sampling were identified as *Stemphylium* spp., *Cladosporium* spp. and *Alternaria* spp., respectively (Appendix A3.6). All the three genera contain species that are recognised plant pathogens.

Table 3.3 Endophytic fungal morphology groups obtained from each sampling and the number of isolates collected in each morphology group. The total number of fungal isolates collected from each sampling is presented in brackets.

Morphology groups	Number of isolates collected in each morphology group			
	LU (170)	HBHV (316^{h}/ 25^{c})	M1 (725)	M2 (60)
Gf1	0	2^{h}	12	1
Gf2	0	0^{h}	2	1
Gf3	8	12^{h}	23	3
Gf4	0	2^{h}	1	0
Gf5	27	1^{h} /1^{c}	194	3
Gf6	0	0^{h}	26	0
Gf7	0	0^{h}	3	0
Gf8	0	0^{h}	1	0
Gf9	0	0^{h}	2	0
Gf10	0	1^{h}	15	1
Gf11	0	33^{h}/3^{c}	39	1
Gf12	0	1^{h}/1^{c}	1	0
Gf13	0	5^{h}/3^{c}	3	0
Gf14	0	1^{h}	1	0
Gf15	0	5^{h}	8	0
Gf16	0	15^{h}/1^{c}	1	0
Gf17	0	33^{h}/4^{c}	3	5
Gf18	0	1^{h}/2^{c}	2	0
Gf19	0	0^{h}	2	0
Gf20	109	149^{h}/3^{c}	333	25
Gf21	0	6^{h}	2	4
Gf22	7	2^{h}	1	8
Gf23	0	1^{h}	1	0
Gf24	0	5^{h}	4	0
Gf25	1	0^{h}	11	2
Gf26	0	0^{h}	4	0
Gf27	0	0^{h}	1	0
Gf28	0	3^{h}	1	0
Gf29	0	0^{h}	3	0
Gf30	0	21^{h}/3^{c}	1	0
Gf31	0	0^{h}	1	0
Gf32	18	13^{h}/4^{c}	20	2
Gf33	0	0^{h}	3	0
Gf34	0	0^{h}	0	1
Gf35	0	0^{h}	0	2
Gf36	0	0^{h}	0	1
Gf37	0	1^{h}	0	0
Gf38	0	2^{h}	0	0
Gf39	0	1^{h}	0	0

LU- Lincoln University research orchard, HBHV- heritage varieties sampling in the Plant & Food Research orchard in Hawke's Bay, M1- Hawke's Bay and Nelson sampling in spring 2015, and M2- Nelson sampling in autumn 2016. ^{h} fungal isolates were from the 35 heritage varieties, ^{c} fungal isolates were from the four commercial varieties which were also sampled in the HBHV sampling.

Figure 3.1 Representative endophytic fungal isolates grown on PDA for each of the 39 groups classified based on colony morphology.

3.3.2 Culturable fungi community analysis

3.3.2.1 Culturable fungi community from heritage and commercial varieties in the HBHV sampling

There was a significant effect of variety on the culturable fungal community isolated in the HBHV sampling (PERMANOVA test; $p = 0.001$; Table 3.4), with the culturable fungal communities separating into two major groups, Group a and Group b (Figure 3.2). The fungal communities from eleven heritage varieties ('Tydeman's Late Orange',' Adams Pearmain', 'Priscilla', 'Laxton's Superb,' 'Granny Smith', 'Lady Sudeley', 'Scarlet Pimpernel', 'Newtown Pippin', 'Sunset, Cox's' 'Orange Pippin ELMA 2' and 'Monty's Surprise') clustered in Group a, and were significantly different to the fungal communities from the other 24 heritage varieties and the four commercial varieties which clustered in Group b (solid line indicates significant difference at $p = 0.05$; Figure 3.2). There was greater morphotype diversity in Group b (17 fungal morphotype groups) than Group a (4 fungal morphotype groups), with all the morphotypes recovered from the varieties in Group a also recovered from the varieties in Group b (Table 3.4). Fungal morphotypes Gf10, Gf14, Gf22, Gf23, Gf37, Gf38 and Gf39 (1-2 isolates each) did not contribute to the separation of the two groups.

Most of the separation was attributed to Gf20 and Gf3 in Group a and Gf20, Gf11, Gf17 and Gf30 in Group b, respectively (percent contribution > 10.0%; Table 3.4). Since the bark was removed prior to plating for 19 out of 28 varieties (68%) in Group b, whilst the bark was left on the plant material plated for all the 11 varieties in Group a, this factor was likely to be associated with the difference in the culturable fungal communities (isolation of each plant material with/without bark shown in Appendix A3.1). A SIMPROF cluster analysis for the 19 varieties (15 heritage varieties and 4 commercial varieties) where the bark was removed prior to isolation showed no significant difference in the culturable fungi community of different varieties (Appendix A3.7).

Morphotype Gf20 (*Alternaria* spp.) was the most common fungal group recovered from the 39 apple varieties (recovery frequency = 33/39; Table 3.4). It was isolated irrespective of whether the bark was attached or not. There was a consistency in the percent contribution of a fungal morphotype to the two apple variety groups and the recovery frequency of the morphotype from the total varieties in that clustered group (Table 3.4).

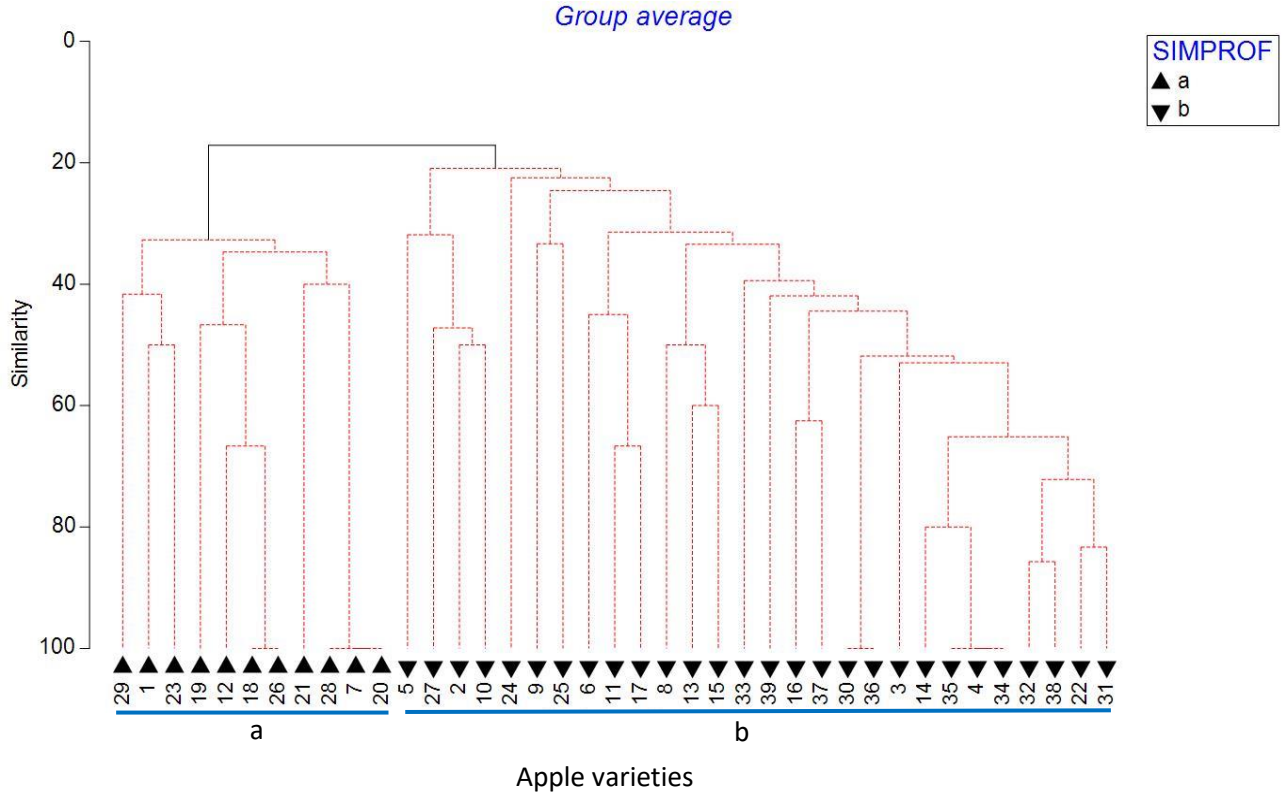

Figure 3.2 SIMPROF profile using group average cluster analysis of the endophytic culturable fungi groups similarities from the presence and absence data of each morphotype recovered from the 35 heritage varieties and four commercial varieties. Heritage varieties: 1. 'Adams Pearmain', 2. 'Cortland', 3. 'Ellison's Orange', 4. 'Api rose', 5. 'Benoni', 6. 'Cornish Aromatic', 7. 'Cox's Orange Pippin ELMA 2', 8. 'Early Strawberry', 9. 'Egremont Russet', 10. 'Esopus Spitzenberg', 11. 'Golden Pippin', 12. 'Granny Smith', 13. 'Hetlina', 14. 'Idared', 15. 'Ingrid Marie', 16. 'Erwin Baur', 17. 'King David', 18. 'Lady Sudeley', 19. 'Laxton's Superb', 20. 'Monty's Surprise', 21. 'Newtown Pippin', 22. 'Northern Spy', 23. 'Priscilla', 24. 'Red Astrachan', 25. 'Robusta 5', 26. 'Scarlet Pimpernel', 27. 'Spartan', 28. 'Sunset', 29. 'Tydeman's Late Orange', 30. 'Winston', 31. 'Grimes Golden', 32. 'Mr Gladstone', 33. 'Nonpareil', 34. 'Orin', 35. 'Orleans Reinette', and commercial varieties: 36. 'Braeburn', 37. 'Gala', 38. 'Royal Gala' and 39. 'Royal Gala Ten Hove' (solid line indicates significant difference at p = 0.05).

Table 3.4 Contribution of the culturable fungal morphological groups to the average similarity within the two apple variety groups from the Hawke's Bay heritage variety sampling determined by SIMPER analysis.

Group[&]	Fungal morphotypes	Genera[#]	Percent contribution in groups	Frequency of recovery from varieties[$]
a	Gf20	*Alternaria* spp.	79.5%	11/11
	Gf3	*Epicoccum* spp./ *Ceratocystis* spp./ *Glonium* spp./ *Saperda* spp.	12.5%	5/11
	Gf16	Unidentified	7.4%	4/11
	Gf24	Unidentified	0.7%	2/11
b	Gf20	*Alternaria* spp.	27.5%	22/28
	Gf11	*Cladosporium* spp.	20.4%	20/28
	Gf17	Unidentified	20.4%	20/28
	Gf30	Unidentified	19.4%	19/28
	Gf32	Unidentified	6.2%	12/28
	Gf16	Unidentified	2.1%	7/28
	Gf13	Unidentified	1.3%	6/28
	Gf15	Unidentified	1.0%	5/28
	Gf3	*Epicoccum* spp./ *Ceratocystis* spp./ *Glonium* spp./ *Saperda* spp.	0.6%	4/28
	Gf21	*Diaporthe* spp.	0.5%	3/28
	Gf18	*Xylariaceae* sp.	0.3%	3/28
	Gf24	Unidentified	0.1%	2/28
	Gf28	Unidentified	0.1%	2/28
	Gf12	Unidentified	0.1%	2/28
	Gf1	*Epicoccum* spp.	0.1%	2/28
	Gf5	*Stemphylium* spp.	0.1%	2/28
	Gf4	Unidentified	0.1%	2/28

[#]Fungal identification based on the results of Section 3.3.4. [$]The number of apple varieties of the total for that group where the fungal morphotype was recovered. [&]Group a and Group b apple varieties clustered by SIMPROF analysis in Figure 3.2.

3.3.2.2 Culturable fungi community associated with different orchard factors in the M1 and M2 samplings

The composition of culturable fungi community in apple shoots collected from 23 blocks (with six blocks sampled in both spring and autumn) clustered into six groups by SIMPROF test ($p \leq 0.05$, Figure 3.3). The fungal communities from the autumn sampling from Nelson (M2) clustered into three groups; M2-S1RG(Nel) and M2-S2RG(Nel) in Group a, M2-S1BN(Nel) and M2-S2BN(Nel) in Group b and M2-S4RG(Nel) and M2-S4BN(Nel) in Group d, along with M1-S7RG-Org(HB) in Group c which were significantly different to the fungal communities in the spring sampling (M1) which clustered into Group e and Group f. Based on the ANOSIM one-way analysis, only season

significantly influenced the culturable fungi community in the test apple blocks (Global R = 0.496, p = 0.001; Table 3.5). Region (Hawke's Bay and Nelson), orchard site (site 1-9), variety '(Royal Gala', 'Braeburn' and 'Scifresh'), management practice (organic and conventional) and infection level (high and low *N. ditissima* level) did not significantly affect the fungal community structure (p > 0.05; Table 3.5).

Three fungal morphotypes, Gf20 (*Alternaria* spp.), Gf5 (*Stemphylium* spp.) and Gf11 (*Cladosporium* spp.) contributed the most to the fungal community structure of the spring (M1) samplings (percent contribution > 10.0%; Table 3.6), verifying they were the three largest groups of culturable fungi in the M1 samplings (Section 3.3.1). In contrast, morphotypes Gf20, Gf22 (unidentified) and Gf3 (*Epicoccum* spp./ *Ceratocystis* spp./ *Glonium* spp./ *Saperda* spp.) mainly contributed to the fungal community structure for the autumn sampling (M2) (percent contribution > 10.0%; Table 3.6). *Alternaria* spp. was the most common fungal group, being recovered from apple tissue samples from all 23 orchard blocks at both sample times (Table 3.6). It was isolated from both apple leaf and stem samples (data not shown). Morphotype Gf22 was only recovered in the autumn sampling.

Season, together with region, variety and infection level, did not show effect on the culturable fungal community when analysing samples from the selected orchard blocks (Appendix A3.8).

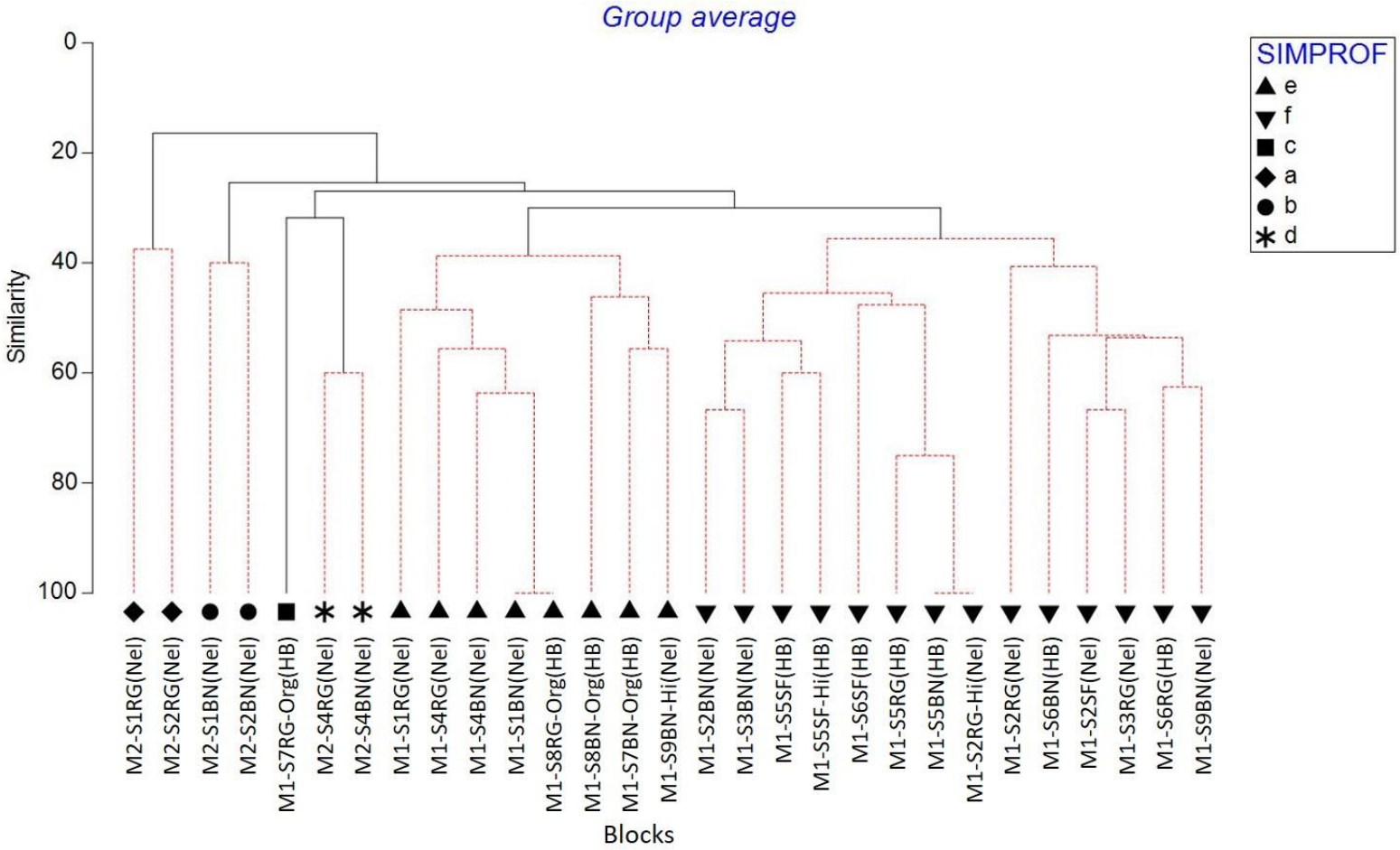

Figure 3.3 SIMPROF profile using group average cluster analysis of the culturable endophytic fungal morphological group similarities from presence and absence data from 23 apple blocks from the M1 and M2 samplings. S signifies site. RG, BN and SF signify 'Royal Gala', 'Braeburn' and 'Scifresh', respectively. Org and Hi signifie organic and high European canker infection (≥ 50% infection). Nel and HB signify Nelson and Hawke's Bay, respectively (solid line indicates significant difference at $p = 0.05$).

Table 3.5 Analysis of similarities (ANOSIM) showing the strength of each environmental factor (R value) and statistic difference (*p* value) in culturable fungi community affected by six factors.

Environmental factors	Global R	*p*
Region	-0.011	0.514
Site	0.068	0.208
Cultivar	-0.003	0.474
Season	0.496	0.001**
Management practice	0.204	0.086
Infection level	-0.150	0.797

** highly significantly different ($p \leq 0.005$).

Table 3.6 Contribution of culturable fungal groups to average similarity within spring and autumn samplings by SIMPER test.

Seasons	Fungal groups	Species[#]	Percent contribution in the seasons	Frequency of recovery from blocks
Spring	Gf20	*Alternaria* spp.	35.0%	23/23
	Gf5	*Stemphylium* spp.	31.8%	22/23
	Gf11	*Cladosporium* spp.	11.1%	14/23
	Gf6	Unidentified	6.2%	11/23
	Gf3	*Epicoccum* spp./ *Ceratocystis* spp./ *Glonium* spp./ *Saperda* spp.	4.5%	10/23
	Gf25	*Chaetomium* spp.	3.2%	7/23
	Gf10	*Biscogniauxia* spp.	2.5%	7/23
	Gf32	Unidentified	2.1%	7/23
	Gf1	*Epicoccum* spp.	2.0%	7/23
	Gf15	Unidentified	0.9%	5/23
	Gf13	Unidentified	0.2%	3/23
	Gf7	Unidentified	0.1%	2/23
	Gf33	Unidentified	0.1%	2/23
	Gf2	*Epicoccum* spp.	0.1%	2/23
	Gf24	Unidentified	0.1%	2/23
	Gf9	Unidentified	0.1%	2/23
	Gf29	*Phlyctema* spp./ *Neofabraea* spp.	0.1%	2/23
Autumn	Gf20	*Alternaria* spp.	45.3%	6/6
	Gf22	Unidentified	14.5%	4/6
	Gf3	*Epicoccum* spp./ *Ceratocystis* spp./ *Glonium* spp./ *Saperda* spp.	11.6%	3/6
	Gf17	Unidentified	7.7%	3/6
	Gf5	*Stemphylium* spp.	7.0%	3/6
	Gf21	*Diaporthe* spp.	7.0%	3/6
	Gf35	Unidentified	2.9%	2/6
	Gf25	*Chaetomium* spp.	2.0%	2/6
	Gf32	Unidentified	2.0%	2/6

3.3.3 Dual culture screening for biocontrol activity of culturable endophytic bacteria and fungi against *N. ditissima* ICMP14417

3.3.3.1 Dual culture assays for endophytic bacteria

In the pilot study, of the 87 endophytic bacteria isolates recovered in the LU sampling, three isolates showing type E and F interactions had antagonistic activity and were selected for the secondary dual culture assay (Table 3.7). In the primary dual culture assay for endophytic bacteria derived from the M1, M2 and HBHV samplings, 23 bacterial isolates with interaction types D, E, and F were selected for the secondary dual culture assay (Table 3.7). Thus, a total of 26 bacterial isolates were selected for the secondary dual culture assay (Table 3.8).

The interaction type showed by the test bacterial isolates in the secondary dual culture assay was consistent with that observed in the primary dual culture assay for most of the isolates, except for five isolates showing type D in the primary dual culture assay and type E/F in the secondary dual culture assay (Table 3.8). In addition, inoculating only one endophytic bacterial isolate per plate in the secondary dual culture assay verified that there was no interaction effect between the different bacterial isolates. Of the 26 bacterial isolates assayed, 18 inhibited the growth of *N. ditissima* ICMP14417 by > 20% (20.0-81.0%) (Figure 3.4) and were selected for PCR identification.

Overall, of the 1004 bacteria tested, 1.8% (n = 18) inhibited the radial growth of *N. ditissima* ICMP1447 by > 20% in the secondary dual culture assay (Table 3.8). Of these 18 antagonistic bacteria, 16 isolates were from commercial cultivars ('Royal Gala' = 9, 'Braeburn' = 5 and 'Scifresh' = 2), with a frequency of 2% (16 out of 870 isolates from commercial cultivars). Two isolates were from the heritage variety 'Grimes Golden', with a frequency of 1.5% (two out of 134 isolates from heritage varieties). In the autumn sampling (M2) five out of 58 isolates (9%) were antagonistic, with four from 'Royal Gala' (14%, four out of 29 isolates) and one from 'Braeburn' (3%, one out of 29 isolates). In the spring sampling (M1), the frequency was 1% (eight out of 725 isolates), with two from 'Royal Gala', four from 'Braeburn' and two from 'Scifresh' (Table 3.8). For both of 'Royal Gala' and 'Braeburn' collected from six sites (site 1- site 6) in the M1 sampling, recovery frequency of antagonistic bacterial isolates was two out of 148 isolates for 'Royal Gala' (1.4%) and four out of 147 isolates for 'Braeburn' (2.7%). In addition, three antagonistic bacteria were isolated from high European canker infection blocks (infection rate ≥ 50%), but all inhibited the growth of *N. ditissima* ICMP14417 by less than 20.0%. All the 18 bacterial isolates selected for further work were from IFP managed orchards (Table 3.8).

Table 3.7 Number of bacterial isolates for each interaction type in the primary dual culture assay with *Neonectria ditissima* ICMP14417 for each sampling. The total number of bacteria tested for each sampling are presented in brackets.

Interaction types	Number of tested bacterial isolates at each sampling			
	LU (87)	HBHV (134)	M1 (725)	M2 (58)
Type A	84	120	479	45
Type B		10	199	6
Type C		0	32	0
Type D		4	9	2
Type E	1	0	4	2
Type F	2	0	1	1

LU- Lincoln University research orchard, HBHV- heritage varieties sampling in the Plant & Food Research orchard in Hawke's Bay, M1- Hawke's Bay and Nelson sampling in spring 2015, and M2- Nelson sampling in autumn 2016.

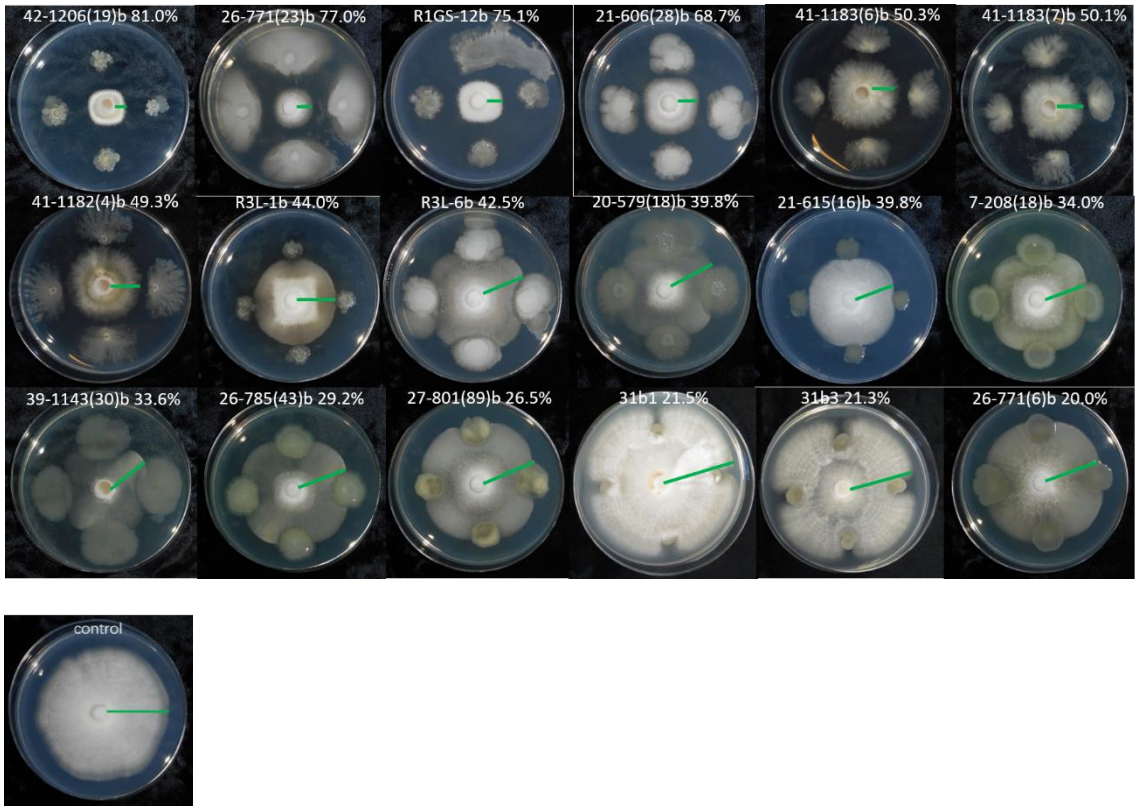

Figure 3.4 Inhibition of *Neonectria ditissima* ICMP14417 colony growth in dual culture with the 18 selected endophytic bacteria in the secondary dual culture assay. The green line indicates the measurement of the radial growth of *N. ditissima*. (Note: some dual culture plates showed higher percent inhibition but with larger radial growth of *N. ditissima* as percent inhibition was calculated compared with their respective negative control).

Table 3.8 Interaction type of the 26 selected bacterial isolates tested in the primary and secondary dual culture assays and percent inhibition of *Neonectria ditissima* isolate ICMP14417 colony growth in the secondary dual culture assay. Number of bacteria tested for each sampling are presented in brackets.

Sampling	Bacterial isolate	Primary assay	Secondary assay		Region origin	Season origin	Variety origin	Infection level
		Interaction type	Interaction type	Inhibition				
LU (87)	R3L-1b*	F	F	44.0%	Lincoln University	Autumn	'Royal Gala'	none
	R3L-6b*	E	E	42.5%			'Royal Gala'	
	R1GS-12b*	F	F	75.1%			'Royal Gala'	
HBHV (134)	16b1	D	D	18.4%	Hawke's Bay	Spring	'Erwin Baur'	none
	31b1*	D	D	21.5%			'Grimes Golden'	
	31b2	D	D	19.5%			'Grimes Golden'	
	31b3*	D	D	21.3%			'Grimes Golden'	
M1 (725)	3-96 (46)b	D	E	7.4%	Nelson	Spring	'Royal Gala'	low
	7-208 (18)b*	E	E	34.1%			'Scifresh'	low
	20-579 (18)b*	E	E	39.8%			'Braeburn'	low
	21-615 (16)b*	D	D	39.8%			'Royal Gala'	low
	21-606 (28)b*	F	F	68.6%			'Royal Gala'	low
	26-771 (24)b	D	D	17.2%	Hawke's Bay	Spring	'Braeburn'	low
	26-771 (6)b*	D	E	20.0%			'Braeburn'	low
	26-771 (23)b*	D	F	77.0%			'Braeburn'	low
	26-785 (43)b*	E	E	29.2%			'Braeburn'	low
	27-801 (89)b*	E	E	26.5%			'Scifresh'	low
	27-812 (91)b	D	D	13.0%			'Scifresh'	low
	29-896 (57)b	D	D	8.6%			'Scilate'	high
	29-896 (28)b	D	D	17.6%			'Scilate'	high
	29-908 (17)b	D	D	19.3%			'Scilate'	high
M2 (58)	39-1143(30)b*	D	E	33.6%	Nelson	Autumn	'Royal Gala'	low
	41-1183(7)b*	E/F	F	50.1%			'Royal Gala'	low
	41-1183(6)b*	D	F	50.3%			'Royal Gala'	low
	41-1182(4)b*	E/F	F	49.3%			'Royal Gala'	low
	42-1206(19)b*	F	F	81.0%			'Braeburn'	low

* Eighteen selected bacterial isolates for further assay. LU- Lincoln University research orchard, HBHV- heritage varieties sampling in the Plant & Food Research orchard in Hawke's Bay, M1- Hawke's Bay and Nelson sampling in spring 2015, and M2- Nelson sampling in autumn 2016. All the sampling sites were managed by IFP.

3.3.3.2 Dual culture assays for endophytic fungi

Primary dual culture assay

In the primary dual culture assay for endophytic fungi, the interaction type was observed 22 days after inoculation. Twenty-two out of the 87 isolates were type C/D (antagonistic) in the initial primary dual culture assay (Table 3.9). Types A and B (not antagonistic) contained one and 64 isolates, respectively (data not shown).

In the initial primary dual culture assay, a representative number (n = 11) of fungi from the three dominant morphology groups (Gf5, Gf11 and Gf20) which were identified as potential plant pathogens (Section 3.3.1) did not inhibit the growth of *N. ditissima* ICMP14417 (with type B interaction; data not shown). Therefore, all the isolates from the three dominant fungal groups were excluded from any further analysis.

In the primary dual culture assays, four isolates 20-585(2)f, 18-538f, 27-806f and 1-27f which showed type D in the initial primary dual culture assay showed type B interaction in the repeated primary dual culture assay and were excluded from any other assessments. In the repeated primary dual culture assay, 14 isolates had a consistent interaction type (C or D) with the initial primary dual culture assay and three isolates had potential type C/D (Table 3.9). They were selected for the secondary dual culture assay.

Secondary dual culture assay

The secondary dual culture assay was carried out for the 18 isolates selected from the primary dual culture assay (Table 3.9). As there was a large difference in the growth rate of the different fungal endophyte isolates, all were divided into three groups based on their growth rate on PDA, including nine isolates as fast-growing fungi (approx. 5-6 cm diameter at three days), six isolates as medium growing fungi (approx. 5-6 cm diameter at 12 days) and three isolates as slow-growing fungi (approx. 4-5 cm diameter at 24 days) (Table 3.9). In the secondary dual culture assay, 12 isolates showed consistent results as the primary dual culture with six isolates confirmed as type C and six isolates confirmed as type D (Table 3.9, Figure 3.5). Interaction type was not affected by the different growth rate of the fungal isolates. The interaction type of the five isolates (34-1029(1)f, 34-1026(3)f, 1-35f, 2-57f and 2-66f) were not confirmed in the secondary dual culture assay, because the replicates did not show a consistent interaction type. All the 17 isolates showing confirmed or potential type C/D in the secondary dual culture assay were included in PCR identification.

A total of 17 isolates representing 10 morphotypes were selected from the 87 fungal isolates in the primary and secondary dual culture assays. Eleven of the fungal isolates were selected from the 59 tested isolates (19%) recovered from commercial varieties sampled in 25 IFP-managed blocks from 12 sites (Appendix A3.2), with two from 'Royal Gala', three from 'Braeburn', one from 'Scifresh' and five from 'Scilate'. Six isolates were selected from the 28 tested isolates (21%) from commercial varieties sampled in four organic blocks from two sites (Appendix A3.2), with one from 'Royal Gala' and five from 'Braeburn'. Additionally, 14 isolates were from low infection level apple blocks (infection rate ≤ 30%), two from high infection blocks (infection rate ≥ 50%) and one from medium infection blocks (30% < infection rate < 50%).

In summary, 18 endophytic bacteria (inhibition effect > 20.0%) and 18 endophytic fungi (12 with confirmed inhibition type) were selected for identification by PCR sequencing.

Table 3.9 Interaction type of the selected fungal endophytic isolates against *Neonectria ditissima* tested in the primary and secondary dual culture assays and details of their origin. The total number of antagonistic fungal isolates tested in each dual culture assay is presented in brackets.

Fungal isolate	Interaction type			Region origin	Management practice	Variety origin	*N. ditissima* infection level
	Primary assay (initial, n = 22)	Primary assay (repeat, n = 22)	Secondary assay (n = 17)				
1-27f (F)	D	B	not tested	Nelson	IFP	'Scilate'	high
1-35f (F)	D	B/D	B/D	Nelson	IFP	'Scilate'	high
1-38f (F)	C	C	C	Nelson	IFP	'Scilate'	high
2-51f (F)	C	C	C	Nelson	IFP	'Scilate'	low
2-57f (F)	D	B/D	B/D	Nelson	IFP	'Scilate'	low
2-66f (F)	B/C/D	B/C	B/C	Nelson	IFP	'Scilate'	low
3-73f (S)	D	D	D	Nelson	IFP	'Royal Gala'	low
4-105(2)f (M)	D	D	D	Nelson	IFP	'Braeburn'	low
6-176f (M)	C	C	C	Nelson	IFP	'Royal Gala'	low
10-283(1)f (F)*	D	contaminated					
14-X(2)f (F)	C	C	C	Nelson	IFP	'Scifresh'	medium
18-538f (M)	D	B	not tested	Nelson	IFP	'Scifresh'	high
20-578f (F)	C	C	C	Nelson	IFP	'Braeburn'	low
20-585(2)f (F)	D	B	not tested	Nelson	IFP	'Braeburn'	low
20-594f (F)	C	C	C	Nelson	IFP	'Braeburn'	low
27-806f (S)	D	B	not tested	Hawke's Bay	IFP	'Scifresh'	low
34-1026(3)f (M)	D	D	B/D	Hawke's Bay	Organic	'Braeburn'	low
34-1029(1)f (M)	D	D	B/D	Hawke's Bay	Organic	'Braeburn'	low
35-1049(2)f (M)	D	D	D	Hawke's Bay	Organic	'Royal Gala'	low
36-1072f (S)	D	D	D	Hawke's Bay	Organic	'Braeburn'	low
36-107Xf (S)	D	D	D	Hawke's Bay	Organic	'Braeburn'	low
36-1073(1)f (M)	D	D	D	Hawke's Bay	Organic	'Braeburn'	low

F, M and S represent fast-growing fungi, medium-growing fungi and slow-growing fungi, respectively. *Isolate 10-283(1)f was contaminated after the primary dual culture assay.

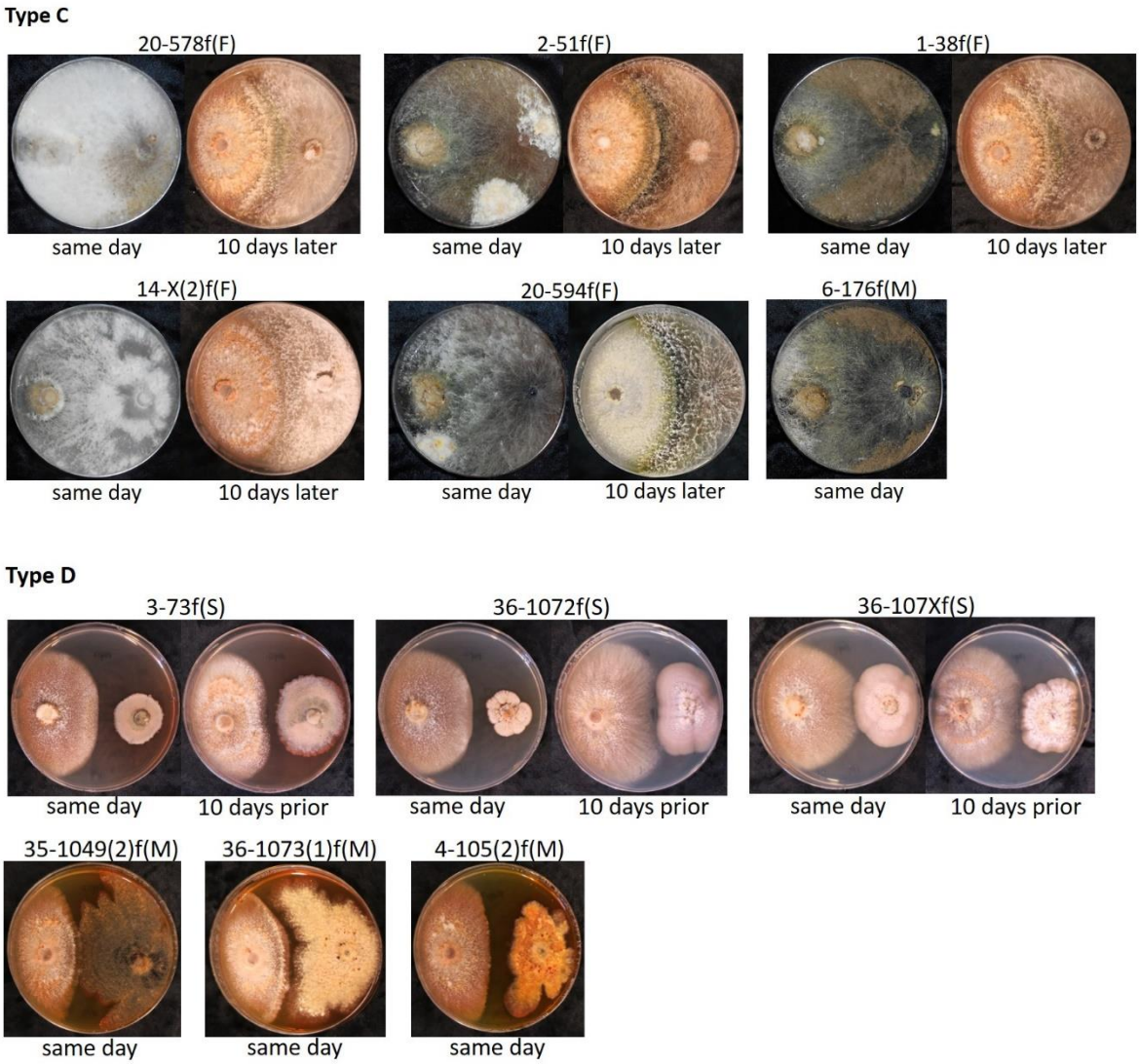

Figure 3.5 Inhibition of *Neonectria ditissima* ICMP14417 (left colony) colony growth by 12 selected endophytic fungi (right colony) with confirmed antagonistic effect (F, M and S represent fast-growing fungi, medium-growing fungi and slow-growing fungi, respectively). Inoculations were done on the same day for medium-growing fungi, or 10 days prior or after for slow and fast-growing fungi, respectively.

3.3.4 Identification of selected bacterial and fungal isolates by PCR sequencing

3.3.4.1 Endophytic bacteria

The phylogenetic tree built with approximately 400 bp of 16S rRNA gene sequences showed that isolates 31b3, 31b1, 7-208(18)b, 26-785(43)b, 39-1143(30)b, 20-579(18)b, 21-615(16)b and 26-771(6)b clustered together with *Pseudomonas* spp. from NCBI GenBank, and isolates 42-1206(19)b, R3L-1b, 26-771(23)b, 21-606(28)b, 41-1183(7)b, 41-1183(6)b, 41-1182(4)b and R1GS-12b clustered together with *Bacillus* spp. from NCBI GenBank. Isolates 27-801(89)b and R3L-6b

were both in a separate cluster, but they belonged to the genera of *Pseudomonas* and *Bacillus*, respectively. All the 18 isolates were separate from the outgroup sequence of *Tychonema bourrellyi* (Figure 3.6).

Sequences of the 18 selected antagonistic bacterial isolates (~600 bp-1400 bp) were blasted in the NCBI GenBank and showed that nine isolates were identified to be *Bacillus* spp. and nine isolates as *Pseudomonas* spp. (Appendix A3.9; A3.11), consistent with the result of the NJ tree. Representatives of the closest relatives of the 18 bacterial isolates obtained from GenBank are listed in Appendix A3.9. Three isolates 42-1206(19)b, R1GS-12b and R3L-1b were identical or highly similar to *B. subtilis*, *B. velezensis* and *B. amyloliquefaciens* and were designated as *Bacillus* sp. 1, *Bacillus* sp. 2 and *Bacillus* sp. 3, respectively. Three isolates 26-771(23)b, 21-606(28)b and R3L-6b had close similarity to *B. thuringiensis*, *B. cereus* and *B. toyonensis* and were designated as *Bacillus* sp. 4, *Bacillus* sp. 5 and *Bacillus* sp. 6, respectively. Three isolates 41-1183(6)b, 41-1183(7)b and 41-1182(4)b were identical or highly similar to *B. horneckiae*, *B. firmus* and/or *B. foraminis* and were designated as *Bacillus* sp. 7, *Bacillus* sp. 8 and *Bacillus* sp. 9, respectively. Isolates 20-579(18)b, 7-208(18)b, 39-1143(30)b, 27-801(89)b, 31b1 and 31b3 were 99%-100% similar to *P. fluorescens* and were designated as *Pseudomonas* sp. 1 to *Pseudomonas* sp. 6, respectively. The six isolates also had highly similarity to other *Pseudomonas* species, such as *P. orientalis* (20-579(18)b), *P. lurida* (7-208(18)b), *P. azotoformans* (31b1), *P. rhodesiae* (31b1 and 31b3), *P. brenneri* (27-801(89)b) and *P. koreensis* (39-1143(30)b). Isolate 26-785(43)b had 99% similarity to *P. poae* and was designated as *Pseudomonas* sp. 7. Isolates 21-615(16)b and 26-771(6)b, designated as *Pseudomonas* sp. 8 and *Pseudomonas* sp. 9, respectively, showed 99% similarity to *P. syringae* which are reported to be plant pathogens on a wide range of plants (Bender et al., 1999; Buell et al., 2003). *Pseudomonas syringae* pv. *actinidiae* is the causal agent of bacterial canker of kiwifruit in New Zealand (Everett et al., 2011). Therefore, isolates 21-615(16)b and 26-771(6)b were excluded from the further study. All other bacterial isolates identified as not being potential plant pathogens were selected for the further study.

Among the five antagonistic isolates obtained from the main sampling in autumn (M2), four *Bacillus* isolates and one *Pseudomonas* isolate were identified. For the eight antagonistic isolates obtained from the main sampling in spring (M1), two *Bacillus* isolates and six *Pseudomonas* isolates were identified (Appendix A3.9).

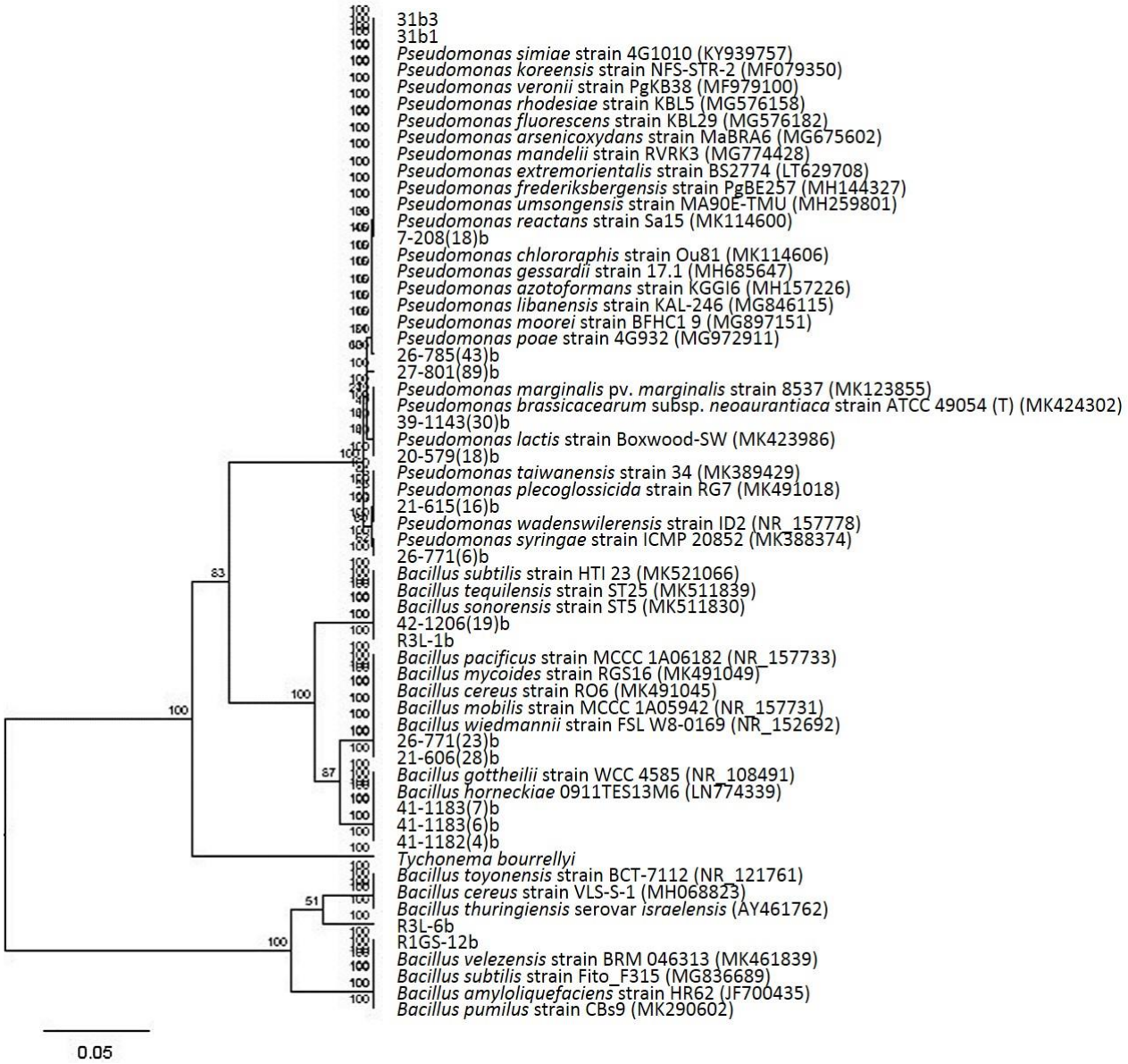

Figure 3.6 Phylogenetic relationship based on partial 16S rRNA gene sequences (~400 bp) of the 18 selected bacterial endophytic isolates showing inhibitory activity to *Neonectria ditissima* ICMP14417 and closely related sequences and an outgroup sequence of *Tychonema bourrellyi* using the UPGMA method in Geneious R10. The numbers at the node are bootstrap values based on 1,000 re-samplings. The bar represents the number of mutations per sequence position.

3.3.4.2 Endophytic fungi

The phylogenetic tree showed that isolates 4-105(2)f, 35-1049(2)f, 36-1073(1)f, 34-1029(1)f and 34-1026(3)f belonged to the genus *Epicoccum*, isolate 6-176f belonged to the genus *Penicillium*, isolate 14-X(2)f belonged to the genus *Diaporthe*, isolates 36-1072f and 36-107Xf belonged to the genus *Phlyctema*, isolates 2-57f, 1-38f and 1-35f belonged to the genus *Chaetomium*, isolates 2-51f, 20-594f, 20-578f, 10-283(1)f and 2-66f belonged to the family *Xylariaceae* or the genus *Biscogniauxia*, and isolate 3-73f was not identified. All the 18 isolates were separated from the outgroup sequence of *Ilyonectria europaea* (Figure 3.7).

For the 18 selected antagonistic fungal isolates, 4 isolates were identified as *Biscogniauxia* spp., 1 isolate as *Diaporthe* sp. (syn. *Phomopsis* sp.), 3 isolates as *Chaetomium* spp., 5 isolates as *Epicoccum* spp., 1 isolate as *Neoseptophoma* sp./*Leptosphaeria* sp./*Coniothyrium* sp., 2 isolates as *Phlyctema vagabunda* (syn. *Neofabraea alba*), 1 isolate as unidentified and 1 isolate as *Penicillium* sp. (Appendix A3.10; A3.11), consistent with the result of the NJ tree except for isolates 3-73f and 20-578f. Representatives of the closest relatives of the 18 fungal isolates obtained from GenBank are listed in Appendix A3.10. Four *Biscogniauxia* spp. 2-51f, 10-283(1)f, 2-66f and 20-594f were designated as *Biscogniauxia* sp. 1 - *Biscogniauxia* sp. 4, respectively. They were highly similar to *Biscogniauxia* sp. 1 ICMP 18828 or *Biscogniauxia* sp. 2 ICMP 18793, both of which were identified as *B. kuntze* and present in New Zealand (International Collection of Micro-organisms from Plants, Landcare Research, Auckland; https://www.landcareresearch.co.nz/resources/collections/icmp). The sequence result demonstrated that isolate 2-51f which was classified into Gf19 should be reclassified into Gf10. One *Diaporthe* sp. 14-X(2)f was designated as *Diaporthe* sp. 1. It was highly similar to *D. viticola*, *D. cynaroidis*, *D. salicicola*, *D. rudis* and *Phomopsis* sp. I405 (syn. *Diaporthe* sp. I405). Three *Chaetomium* spp. 1-38f, 1-35f and 2-57f were designated as *Chaetomium* sp. 1-*Chaetomium* sp. 3, respectively. They were identical or highly similar to *Chaetomium* cf. *cochliodes* MZ-2011 strain CCM F-232, *C. globosum* strain A95 or *C. globosum* isolate BK250A. The sequence result also showed that isolate 1-38f which was classified into Gf19 should be reclassified into Gf25. Five *Epicoccum* spp. 35-1049(2)f, 34-1029(1)f, 36-1073(1)f, 4-105(2)f and 34-1026(3)f were designated as *Epicoccum* sp. 1 - *Epicoccum* sp. 5, respectively. They had 99%-100% similarity to *E. nigrum*. The sequence results showed that morphology groups Gf1, Gf2 and Gf3 from which the five isolates were initially placed should be merged into one group. Isolate 3-73f was designated as *Neoseptophoma* sp./*Leptosphaeria* sp./*Coniothyrium* sp., belonging to the order Pleosporales. Two *Phylctema* spp. 36-1072f and 36-107Xf were designated as *Phlyctema* sp. 1 and *Phlyctema* sp. 2, respectively. They were identical to *Phlyctema vagabunda* (syn. *Neofabraea alba*). The sequence result verified that classification of isolates 36-1072f and 36-

107Xf into the same group (Gf29) was accurate. Isolate 20-578f remained unidentified because it only had 90% similarity to *Xylariaceae* sp. TA1-4-1. One *Penicillium* sp. 6-176f was designated as *Penicillium* sp. 1. It had 99% similarity to *Penicillium ochrochloron*. The sequence result showed that isolate 6-176f should be moved out from Gf10. Isolate 14-X(2)f identified as *Diaporthe* sp. (syn. *Phomopsis* sp.) and isolates 36-1072f and 36-107Xf identified as *Phylctema vagabunda* (syn. *Neofabraea alba*) were omitted from further experiments, because those genera/species have been reported as being plant pathogens.

Some selected bacterial and fungal isolates are likely to be new cultures in New Zealand based on the record in ICMP database (Landcare Research, Auckland). For selected bacterial endophytes, *B. thuringiensis*, *B. subtilis*, *B. cereus*, *P. azotoformans*, *P. fluorescens* and *P. syringae* strains were recorded as present in New Zealand. There are no records of *B. foraminis*, *B. horneckiae*, *B. toyonensis*, *P. brenneri*, *P. orientalis* and *P. poae* in the ICMP database. For selected fungal endophytes, *C. globosum*, *E. nigrum* and *B. kuntze* are recorded as present in New Zealand, whilst *Chaetomium* cf. *cochliodes*, *Neoseptophoma clematidis*, *Neoseptophoma italica* (Appendix A3.10) and *P. ochrochloron* have not been recorded as being present. *Coniothyrium* and *Leptosphaeria* species are recorded as being present but more information is needed to determine if the exact species isolated here are new. However, before these are recorded as new records for New Zealand, further work needs to be carried out to confirm identity.

In summary, 16 endophytic bacterial isolates and 14 endophytic fungal isolates were selected for the remaining experiments.

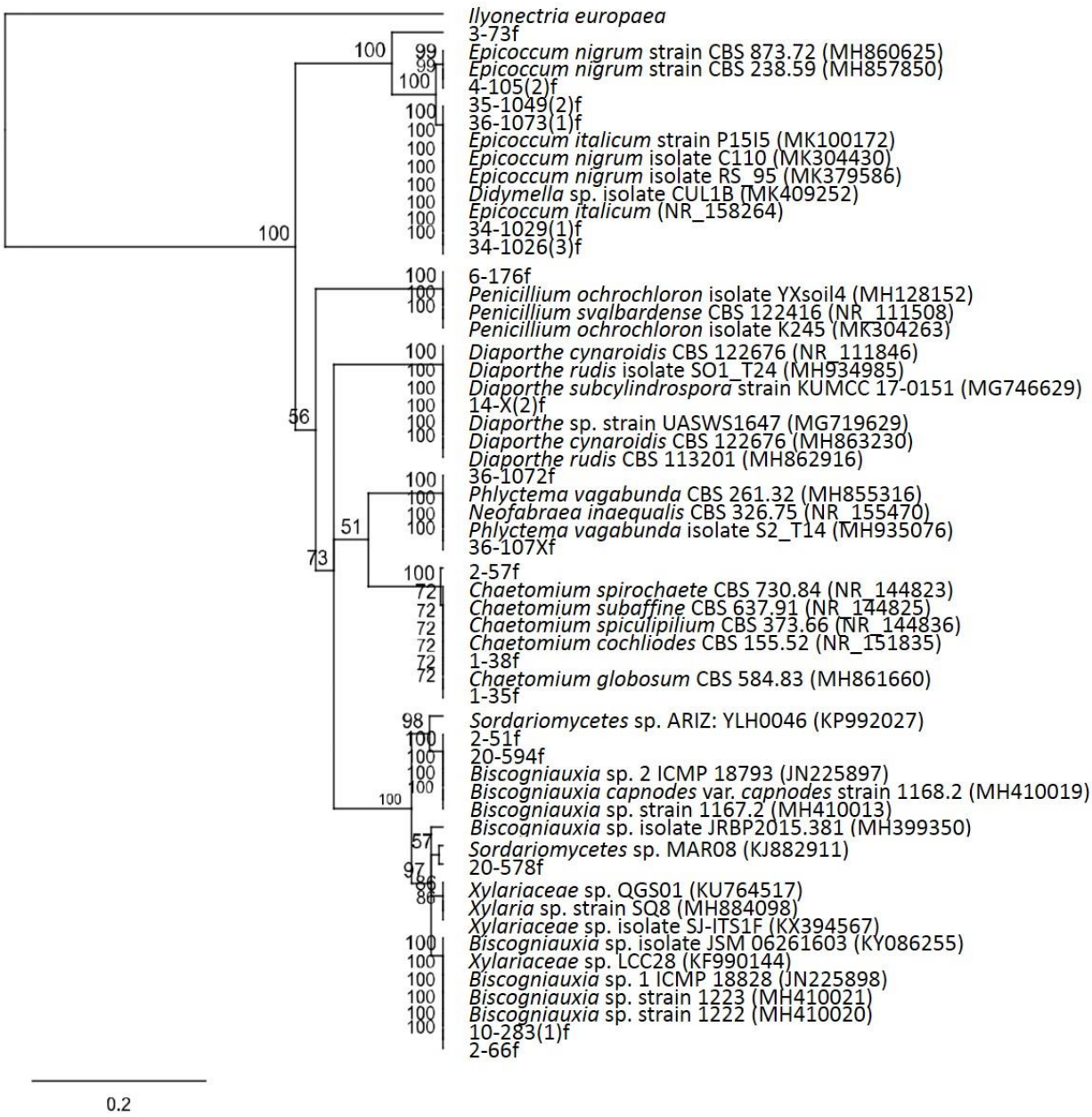

Figure 3.7 Phylogenetic relationship based on sequencing of the ITS gene region of the 18 selected fungal isolates showing inhibitory activity to *Neonectria ditissima* ICMP14417 and closely related sequences and an outgroup sequence of *Ilyonectria europaea* using the UPGMA method in Geneious R10. The numbers at the node are bootstrap values based on 1,000 re-samplings. The bar represents the number of mutations per sequence position.

3.3.5 Comparison of biocontrol activity of the selected endophytic bacteria and fungi against three *N. ditissima* isolates

3.3.5.1 Endophytic bacteria

Sixteen selected bacterial isolates were tested for their inhibition of *N. ditissima* isolates ICMP14417, MW15c1 and RS324p. There was no significant difference in the radial growth of *N. ditissima* ICMP14417, MW15c1 and RS324p across the bacterial isolates ($p = 0.168$, nonparametric Friedman's two-way test; Table 3.10; Appendix A3.12). However, there was significant difference in the radial growth of each *N. ditissima* isolate across the 16 test bacterial isolates ($p \leq 0.001$ for ICMP14417, MW15c1 and RS324p, respectively; nonparametric Kruskal-Wallis one-way test; Table 3.10; Appendix A3.13). The nine bacterial isolates identified as *Bacillus* spp. significantly inhibited the radial growth of the three *N. ditissima* isolates compared with the negative control, with percent inhibition > 50% (Table 3.10). In contrast, the seven *Pseudomonas* spp. did not significantly inhibit the radial growth of the three *N. ditissima* isolates compared with the negative control, with percent inhibition < 50% (Table 3.10). Within the nine *Bacillus* species, there was no significant difference in the antagonistic activity against any of the *N. ditissima* isolates.

3.3.5.2 Endophytic fungi

Six of the 11 tested endophytic fungal isolates showed consistent interaction type against the three *N. ditissima* isolates. Isolates 1-35f and 2-57f had unconfirmed interaction type (type B/C/D) against *N. ditissima* ICMP14417 with C interaction type with the other two *N. ditissima* isolates, and isolates 36-1073(1)f and 4-105(2)f which showed different interaction types against *N. ditissima* ICMP14417 compared to against *N. ditissima* RS324p and MW15c1 (Table 3.11).

Table 3.10 Radial growth (mm) and percent inhibition of three *Neonectria ditissima* isolates (ICMP14417, RS324p and MW15c1) by 16 selected endophytic bacterial isolates in the dual culture plating assay after 17 days growth.

Bacterial isolate	ICMP14417		MW15c1		RS324p	
	Radial growth (mm)	Percent inhibition	Radial growth (mm)	Percent inhibition	Radial growth (mm)	Percent inhibition
Control	32.48 a		33.22 a		32.69 a	
Pseudomonas sp. 27-801(89)b	29.68 ab	8.6%	25.26 abc	24.0%	27.88 a	14.7%
Pseudomonas sp. 31b3	27.02 ab	16.8%	30.32 ab	8.7%	25.15 abc	23.1%
Pseudomonas sp. 31b1	26.58 abc	18.2%	30.73 a	7.5%	26.51 ab	18.9%
Pseudomonas sp. 26-785(43)b	25.37 abcd	21.9%	22.64 abcd	31.9%	25.07 abcd	23.3%
Pseudomonas sp. 20-579(18)b	23.68 abcde	27.1%	19.65 abcdefg	40.8%	19.41 abcdef	40.6%
Pseudomonas sp. 7-208(18)b	21.83 abcdef	32.8%	21.30 abcde	35.9%	20.39 abcde	37.6%
Pseudomonas sp. 39-1143(30)b	19.36 abcdefg	40.4%	19.62 abcdef	40.9%	18.36 abcdefg	43.8%
Bacillus sp. R3L-6b	15.20 bcdefgh	53.2%	13.97 bcdefgh	57.9%	15.06 bcdefgh	53.9%
Bacillus sp. R3L-1b	14.87 bcdefgh	54.2%	15.83 bcdefgh	52.3%	16.32 bcdefgh	50.1%
Bacillus sp. 41-1183(7)b	8.26 cdefgh	74.6%	9.42 cdefgh	71.6%	8.76 cdefgh	73.2%
Bacillus sp. 21-606(28)b	7.40 defgh	77.2%	6.03 efgh	81.9%	7.25 efgh	77.8%
Bacillus sp. 41-1183(6)b	7.14 defgh	78.0%	6.33 defgh	80.9%	7.74 efgh	76.3%
Bacillus sp. 41-1182(4)b	6.52 efgh	79.9%	6.23 defgh	81.3%	8.03 efgh	75.4%
Bacillus sp. 26-771(23)b	4.66 gh	85.7%	4.51 h	86.4%	7.43 efgh	77.3%
Bacillus sp. 42-1206(19)b	4.24 h	87.0%	4.09 h	87.7%	5.58 h	82.9%
Bacillus sp. R1GS-12b	4.19 h	87.1%	5.06 fgh	84.8%	6.20 h	81.0%
p value	< 0.001**		< 0.001**		< 0.001**	
p value (across ICMP14417, MW15c1 and RS324p)	0.168					

The letters after radial growth (mm) are based on the results of the pairwise comparisons using nonparametric Kruskal-Wallis one-way test in SPSS statistics 24. Means followed by the same letter within a column are not significantly different. ** Highly significantly different ($p \leq 0.005$). *p* (across ICMP14417, MW15c1 and RS324p) is based on nonparametric Friedman's two-way test in SPSS statistics 24.

Table 3.11 The inhibition reaction type observed in the interaction between the 11 selected endophytic fungal isolates against three *Neonectria ditissima* isolates (ICMP14417, MW15c1 and RS324p) in the dual culture plating assay after 22 days growth.

Fungal isolate	Genus identification	*Neonectria ditissima* isolate		
		ICMP14417	MW15c1	RS324p
20-578f(F)	Unidentified	C	C	NC
2-51f(F)	*Biscogniauxia* sp.	C	C	C
1-38f(F)	*Chaetomium* sp.	C	C	C
35-1049(2)f(M)	*Epicoccum* sp.	D	D	D
34-1029(1)f(M)	*Epicoccum* sp.	D	D	D
36-1073(1)f(M)	*Epicoccum* sp.	D	B	B
4-105(2)f(M)	*Epicoccum* sp.	D	B	B
34-1026(3)f(M)	*Epicoccum* sp.	D	D	D
3-73f(M)	*Neoseptophoma* sp./ *Leptosphaeria* sp./ *Coniothyrium* sp.	D	D	D
1-35f(F)	*Chaetomium* sp.	B/C/D	C	C
2-57f(F)	*Chaetomium* sp.	B/C/D	C	C

NC-interaction type not clear because the mycelium of both the fungal isolates were white. F, M and S in the brackets after isolate codes mean fast-growing fungi, medium-growing fungi and slow-growing fungi, respectively.

3.3.6 Effect of pathogen presence on production of inhibitory compounds by endophytic bacteria

Radial growth of *N. ditissima* ICMP14417 was significantly decreased ($p \leq 0.05$) with increasing interval days between its inoculation and that of 15 bacterial isolates, except for isolate 31b1 (Table 3.12, Figure 3.8; Appendix A3.14). This indicated that all the endophytic bacteria, apart from isolate 31b1, produced the inhibitory compounds in the absence of the pathogen and they accumulated over time (recorded as secretion). There was no significant difference in the radial growth of *N. ditissima* ICMP14417 when inoculated 1, 4 or 7 days after inoculation with isolate 31b1. Therefore, this indicates that the presence of *N. ditissima* ICMP14417 is required for the production of the inhibitory compounds by this isolate (recorded as induction).

Table 3.12 The effect of inoculation of 16 different endophytic bacteria 1, 4 or 7 days prior to inoculation of *Neonectria ditissima* ICMP14417 on the radial growth of *N. ditissima* ICMP14417 colonies.

Bacterial isolate	Radial growth of *N. ditissima* ICMP14417 (mm) dual-cultured with bacteria inoculated at different intervals			Radial growth (mm) of control	Secretion/ induction[$]
	1 day prior	4 days prior	7 days prior		
Control				32.42	
Ba sp. R1GS-12b	4.87 a	1.45 b	0.00 c		secretion
Ba sp. 42-1206(19)b	3.71 a	1.63 b	0.00 c		secretion
Ba sp. 26-771(23)b	5.14 a	0.19 b	0.00 b		secretion
Ba sp. 41-1182(4)b	5.67 a	1.33 b	0.00 b		secretion
Ba sp. 41-1183(6)b	5.74 a	0.20 b	0.00 b		secretion
Ba sp. 21-606(28)b	5.04 a	1.23 b	0.00 c		secretion
Ba sp. 41-1183(7)b	7.49 a	0.52 b	0.00 b		secretion
Ba sp. R3L-1b	13.05 a	9.39 b	8.38 b		secretion
Ba sp. R3L-6b	13.01 a	8.50 b	7.66 b		secretion
Ps sp. 39-1143(30)b	18.30 a	13.75 b	11.03 c		secretion
Ps sp. 7-208(18)b	20.04 a	18.47 ab	17.89 b		secretion
Ps sp. 20-579(18)b	20.92 a	16.74 b	11.53 c		secretion
Ps sp. 26-785(43)b	24.12 a	20.09 b	14.69 c		secretion
Ps sp. 31b1	28.91 a	28.18 a	27.18 a		induction
Ps sp. 31b3	29.23 a	26.99 b	26.39 b		secretion
Ps sp. 27-801(89)b	22.07 a	18.37 b	13.97 c		secretion

The letters after radial growth (mm) are based on the results of the pairwise comparisons using one-way ANOVA in Minitab 17. Means followed by the same letter are not significantly different. [$] Significant difference in the *N. ditissima* radial growth across the three inoculation times indicates inhibitory compounds are constitutively secreted and referred to as secretion, whereas no significant differences in the *N. ditissima* radial growth across the three inoculation times indicates inhibitory compounds induced by presence of pathogen and is indicated as induction. *Ba* sp. and *Ps* sp. mean *Bacillus* sp. and *Pseudomonas* sp., respectively.

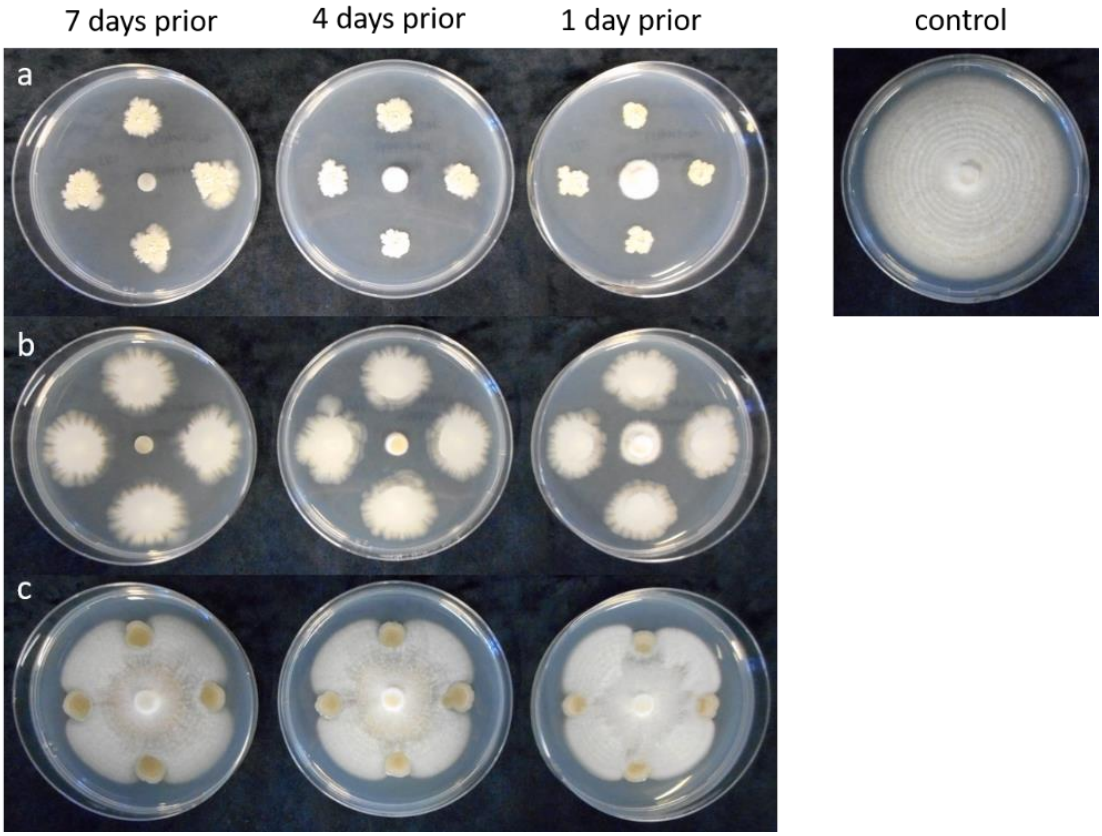

Figure 3.8 Effect of *Neonectria ditissima* (central colony) presence on production of inhibitory compounds by endophytic bacteria. a) and b) indicating isolates 42-1206(19)b and 21-606(28)b produced inhibitory compounds in the absence of *Neonectria ditissima* ICMP14417. c) indicating the production of inhibitory compounds by isolate 31b1 was induced by the presence of *Neonectria ditissima* ICMP14417.

3.3.7 Cell-free filtrate culture assay for selected bacteria and fungi

3.3.7.1 Endophytic bacteria

Cell-free filtrates of *Bacillus* sp. 42-1206(19)b harvested at 38 h amended at 10% and 30% concentrations into WmA, *Bacillus* sp. R1GS-12b harvested at 38 h amended at 30% concentration and *Pseudomonas* sp. 27-801(89)b harvested at 16 h amended at 30% concentration significantly inhibited the radial growth of *N. ditissima* ICMP14417 compared with the negative control, with 56.1%, 64.8%, 45.4% and 43.5% inhibition, respectively (Table 3.13; Appendix A3.15). These did not differ significantly from each other (data not shown). In addition, cell-free filtrates of *Bacillus* sp. 21-606(28)b harvested at 38 h amended at 30% concentration into WmA and *Pseudomonas* sp. 7-208(18)b harvested at both 16 h and 38 h amended at 30% concentration inhibited the radial growth of *N. ditissima* ICMP14417 by over 20%, but were not significantly different to the negative control (Table 3.13).

Pseudomonas sp. 20-579(18)b after 16 h, and three *Bacillus* spp. 41-1182(4)b, 41-1183(6)b and 41-1183(7)b after both 16 h and 38 h had not grown sufficiently and therefore the CF filtrate for these isolates were not collected (Table 3.13).

3.3.7.2 Endophytic fungi

After 22 days incubation of *N. ditissima* ICMP14417, both 1% and 10% concentrations of the CF filtrates from isolates 1-38f (fast-growing isolate), 34-1029(1)f (medium-growing isolate) and 36-1072f (slow-growing isolate) significantly inhibited the radial growth of *N. ditissima* ICMP14417 compared to the negative control (Table 3.14; Appendix A3.16). For isolate 36-1072f, the radial growth of *N. ditissima* ICMP14417 was significantly decreased on agar amended with 10% CF filtrate compared with at 1%. There was no significant difference in the radial growth of *N. ditissima* ICMP14417 on agar amended with 1% and 10% CF filtrates for isolates 1-38f and 34-1029(1)f.

Table 3.13 Radial growth of *Neonectria ditissima* ICMP14417 on Waksman agar (WmA) amended with different concentrations of 16 h and 38 h cell-free (CF) broth culture filtrates from 13 selected endophytic bacterial isolates as compared with radial growth of *N. ditissima* ICMP14417 on unamended WmA after 14 days incubation.

Bacterial isolate	16 h						38 h					
	1%		10%		30%		1%		10%		30%	
	Radial growth (mm)	Percent inhibition	Radial growth (mm)	Percent inhibition	Radial growth (mm)	Percent inhibition	Radial growth (mm)	Percent inhibition	Radial growth (mm)	Percent inhibition	Radial growth (mm)	Percent inhibition
Ba sp. R1GS-12b	29.83	-6.0%	30.38	-7.9%	29.77	-5.7%	30.04	-6.6%	24.56	12.8%	**15.38***	**45.4%**
Ba sp. 42-1206(19)b	27.70	1.7%	28.82	-2.3%	26.54	5.8%	25.83	8.3%	**12.36***	**56.1%**	**9.91***	**64.8%**
Ba sp. 26-771(23)b	28.19	-0.1%	24.20	14.1%	22.73	19.3%	29.11	-3.3%	29.28	-4.0%	29.33	-4.1%
Ba sp. 21-606(28)b	25.62	9.1%	27.77	1.4%	25.64	9.0%	28.13	0.2%	26.96	4.3%	17.25	38.8%
Ba sp. R3L-1b	28.10	0.3%	28.48	-1.1%	27.75	1.5%	27.41	2.7%	27.30	3.1%	27.41	2.7%
Ba sp. R3L-6b	25.41	9.8%	26.69	5.3%	27.17	3.5%	27.50	2.4%	28.61	-1.6%	26.01	7.7%
Ps sp. 39-1143(30)b	28.80	-2.2%	28.67	-1.8%	29.91	-6.2%	29.02	-3.0%	29.79	-5.8%	29.13	-3.4%
Ps sp. 7-208(18)b	26.66	5.4%	27.37	2.9%	22.32	20.8%	28.39	-0.8%	25.83	8.3%	22.44	20.4%
Ps sp. 26-785(43)b	28.65	-1.7%	29.10	-3.3%	28.76	-2.1%	28.73	-2.0%	28.90	-2.6%	27.78	1.4%
Ps sp. 31b1	28.45	-1.0%	28.27	-0.4%	29.34	-4.2%	28.44	-1.0%	26.88	4.6%	28.35	-0.6%
Ps sp. 31b3	28.36	-0.7%	29.16	-3.5%	27.67	1.8%	28.24	-0.2%	29.26	-3.9%	29.71	-5.5%
Ps sp. 27-801(89)b	27.16	3.6%	25.91	8.0%	**15.90***	**43.5%**	29.12	-3.4%	29.06	-3.2%	28.93	-2.7%
Ps sp. 20-579(18)b				NT			28.80	-2.3%	30.50	-8.3%	30.13	-7.0%
Ba sp. 41-1182(4)b				NT						NT		
Ba sp. 41-1183(6)b				NT						NT		
Ba sp. 41-1183(7)b				NT						NT		
Control	28.17											

*Mean values in bold are significantly different compared to the negative control ($p \leq 0.05$) based on one-way Kruskal-Wallis test with pairwise comparison performed in the SPSS statistics 24. *Ba* sp. and *Ps* sp. mean *Bacillus* sp. and *Pseudomonas* sp., respectively. NT means not tested.

Table 3.14 Radial growth of *Neonectria ditissima* ICMP14417 on potato dextrose agar (PDA) amended with different concentrations of cell-free (CF) broth culture filtrates from nine selected endophytic fungal isolates as compared with radial growth of *N. ditissima* ICMP14417 on unamended PDA after 22 days incubation.

Tested fungal isolates	Genus identification	1% CF filtrate	10% CF filtrate	0%
Control				33.6 ab
1-38f(F)	*Chaetomium* sp.	30.8 efgh	31.5 defg	
2-51f(F)	*Biscogniauxia* sp.	33.4 abc	32.9 abcd	
3-73f(S)	*Neoseptophoma* sp./ *Leptosphaeria* sp./ *Coniothyrium* sp.	32.6 abcde	31.9 bcdef	
20-578f(F)	Unidentified	32.5 abcde	33.0 abcd	
34-1026(3)f(M)	*Epicoccum* sp.	32.8 abcd	33.2 abcd	
34-1029(1)f(M)	*Epicoccum* sp.	30.4 fgh	30.0 gh	
35-1049(2)f(M)	*Epicoccum* sp.	33.4 abc	32.2 bcdef	
36-107Xf(S)	*Phlyctema* sp.	34.2 a	32.3 abcde	
36-1072f(S)	*Phlyctema* sp.	31.8 cdefg	29.2 h	

The letters after radial growth (mm) are based on the results of the pairwise comparisons using one-way ANOVA in Minitab 17. Mean values followed by the same letter are not significantly different.

3.3.8 Evaluation of siderophore production by the selected endophytic bacteria and fungi

3.3.8.1 Endophytic bacteria

In the CAS assay, of the 16 tested bacterial isolates the seven bacterial isolates identified as *Pseudomonas* spp. grew well on the CAS media and produced an orange halo of varying width surrounding the isolate colonies, indicating the production of siderophore-type compounds (Table 3.15; Figure 3.9). These seven siderophore positive bacterial isolates had a CAS reaction rate between 0.9 and 2.3 mm/day, with isolates 31b1 and 31b3 showing the strongest siderophore production activity in this assay (CAS reaction rate of '+++', Table 3.15). In contrast, the nine bacterial isolates identified as *Bacillus* spp. showed slight/no growth on the CAS media and did not produce an orange halo surrounding the bacterial isolate colony (Table 3.15). Since the growth of Gram-positive bacteria such as *Bacillus* spp. could be inhibited by the detergent hexadecyltrimethyl-ammonium bromide (HDTMA) used for CAS medium preparation (Schwyn & Neilands, 1987), a modified CAS (MCAS) method described by Milagres et al. (1999) was used for siderophore detection for all the 16 bacterial isolates. This method allowed all 16 bacterial isolates to grow on the culture medium (NA) half of the plate and enabled the analysis of siderophore production on the CAS half of the plate. After 4 days incubation, all the bacterial isolates grew on the NA medium and reached the border line between the two media. Fifteen of the 16 tested bacterial isolates changed the CAS-blue agar to orange, indicating siderophore production (CAS reaction rate ranging from 0.1 to 3.7 mm/day, Table 3.15). *Bacillus* sp. isolate 41-

1182(4)b did not result in any colour change in the CAS-blue agar. In general, *Pseudomonas* spp. showed stronger siderophore production activity than *Bacillus* spp. (Table 3.15). Isolates 31b1 and 31b3 also showed the strongest siderophore production ability in the MCAS assay as seen in the CAS assay. Isolate 27-801(89)b showed a similar siderophore production activity as 31b1 and 31b3 in the MCAS assay (CAS reaction rate scale of '+++', Table 3.15).

3.3.8.2 Endophytic fungi

The MCAS method was also used for evaluating the siderophore production of the 15 selected endophytic fungal isolates. This method allowed all 15 fungal isolates to grow on the culture medium (PDA) half of the plate and enabled analysis of siderophore production on the CAS half of the plate (Figure 3.9). The test fungal isolates varied in the number of days taken to reach the border line, ranging from 8 to 42 days (Table 3.16). Thirteen of the 15 tested fungal isolates were positive for siderophore production, with CAS reaction rate between 0.3 and 1.4 mm/day (Table 3.16). *Penicillium* sp. isolate 6-176f and three of the five *Epicoccum* sp. isolates (34-1029(1)f, 36-1073(1)f and 35-1049(2)f) had stronger siderophore production activity (CAS reaction rate at '++') than the other test isolates (CAS reaction rate at '+'/ '-'). All the *Epicoccum* sp. isolates were positive for siderophore production in this assay (Table 3.16). The *Chaetomium* spp. and *Biscogniauxia* spp. isolates varied in ability to produce siderophores. *Chaetomium* sp. isolates 1-35f and 1-38f were positive for siderophore production (Sid^+), while isolate 2-57f was negative (Sid^-). Similarly, the three isolates identified as *Biscogniauxia* spp. varied in siderophore production with isolates 10-283(1)f, 2-66f and 20-594f being positive for siderophore production, while isolate 2-51f was negative (Table 3.16). For isolates identified as being from the same genus, there was variation shown in the number of days required for the fungal mycelium to cover the culture medium half of the plate. For example, *Chaetomium* sp. isolate 1-38f required 25 days to cover the PDA half of the plate compared to 8 days for *Chaetomium* sp. isolate 1-35f, but there was a similar CAS reaction rate for the two isolates (CAS reaction rate at '+', Table 3.16).

Table 3.15 Siderophore production indicated by blue to orange media colour change determined using the Chrome Azurol S (CAS) assay and modified CAS (MCAS) by the 16 selected endophytic bacterial isolates.

Bacterial isolate	CAS assay				MCAS assay			
	Growth (days)[a]	CAS-blue agar (colour change)[b]	Radius of orange halo (mm)	CAS reaction rate (mm/day)[d]	Growth (days)[a]	CAS-blue agar (colour change)[b]	Distance (mm)[c]	CAS reaction rate (mm/day)[d]
Ps sp. 31b1	3	orange halo	7.0	2.3 (+++)	4	orange (d)	13.2	3.3 (+++)
Ps sp. 31b3	3	orange halo	6.6	2.2 (+++)	4	orange (d)	13.5	3.4 (+++)
Ps sp. 26-785(43)b	3	orange halo	4.7	1.6 (++)	4	orange (l)	4.7	1.2 (++)
Ps sp. 7-208(18)b	3	orange halo	4.2	1.4 (++)	4	orange (d)	4.8	1.2 (++)
Ps sp. 27-801(89)b	3	orange halo	3.0	1.0 (+)	4	orange (d)	14.9	3.7 (+++)
Ps sp. 20-579(18)b	3	orange halo	2.8	0.9 (+)	4	orange (d)	3.6	0.9 (+)
Ps sp. 39-1143(30)b	3	orange halo	2.7	0.9 (+)	4	orange (d)	4.2	1.1 (++)
Ba sp. R3L-1b	3	slow growth	0.0	0.0 (-)	4	orange (l)	3.4	0.9 (+)
Ba sp. R3L-6b	3	no growth			4	orange (l)	1.3	0.3 (+)
Ba sp. R1GS-12b	3	slow growth	0.0	0.0 (-)	4	orange (l)	4.4	1.1 (++)
Ba sp. 26-771(23)b	3	slow growth	0.0	0.0 (-)	4	orange (d)	2.7	0.7 (+)
Ba sp. 21-606(28)b	3	slow growth	0.0	0.0 (-)	4	orange (l)	2.1	0.5 (+)
Ba sp. 41-1183(7)b	3	slow growth	0.0	0.0 (-)	4	orange (l)	0.5	0.1 (±)
Ba sp. 41-1183(6)b	3	slow growth	0.0	0.0 (-)	4	orange (l)	0.7	0.2 (±)
Ba sp. 41-1182(4)b	3	slow growth	0.0	0.0 (-)	4	no change	0.0	0.0 (-)
Ba sp. 42-1206(19)b	3	slow growth	0.0	0.0 (-)	4	orange (l)	3.6	0.9 (+)

[a] Incubation time of bacterial isolates in the CAS and MCAS assays. [b] The CAS-blue agar changed to orange surrounding the bacterial colony in the CAS assay and the CAS-blue agar changed to dark (d) or light (l) orange in the MCAS assay. [c] Distance (mm) is the mean distance of the advance of the colour-change front in the CAS-blue agar, starting from the borderline between the two media. [d] – 0.0 (no colour change); ± 0.1- 0.2; + 0.3-1.0; ++ 1.1-2.0 and +++ 2.0-4.0 mm/day for the orange halo in the CAS assay and the advancement of colour change front in the CAS-blue agar in the MCAS assay. *Ba* sp. and *Ps* sp. refers to *Bacillus* sp. and *Pseudomonas* sp., respectively.

Table 3.16 Siderophore production indicated by blue to orange media colour changed determined using the modified CAS assay (MCAS) by 15 endophytic fungal isolates.

Fungal isolate	Genus	Growth (days)[a]	CAS-blue agar (colour change)	Distance (mm)[b]	CAS reaction rate (mm/day)[c]
1-35f	*Chaetomium* sp.	8	orange (l)	5.9	0.7 (+)
2-57f	*Chaetomium* sp.	8	no colour change	0.0	0.0 (-)
1-38f	*Chaetomium* sp.	25	orange (l)	7.7	0.3 (+)
10-283(1)f	*Biscogniauxia* sp.	15	orange (d)	10.9	0.7 (+)
2-66f	*Biscogniauxia* sp.	15	orange (d)	11.7	0.8 (+)
2-51f	*Biscogniauxia* sp.	30	no colour change	0.0	0.0 (-)
20-594f	*Biscogniauxia* sp.	30	orange (l)	7.6	0.3 (+)
3-73f	*Neosetophoma* sp.	42	orange (l)	19.9	0.5 (+)
20-578f	Unidentified	11	orange (d)	4.3	0.4 (+)
6-176f	*Penicillium* sp.	11	orange (l)	15.8	1.4 (++)
34-1029(1)f	*Epicoccum* sp.	30	orange (d)	38.1	1.3 (++)
36-1073(1)f	*Epicoccum* sp.	30	orange (d)	32.9	1.1 (++)
34-1026(3)f	*Epicoccum* sp.	34	orange (d)	26.0	0.8 (+)
4-105(2)f	*Epicoccum* sp.	27	orange (d)	10.7	0.4 (+)
35-1049(2)f	*Epicoccum* sp.	39	orange (d)	41.2	1.1 (++)

[a] Growth (days) required for the fungal mycelium to cover the culture medium half of the plate. [b] Distance (mm) is the mean distance of the advance of the colour-change front in the CAS-blue agar, starting from the borderline between the two media. [c] The CAS-blue agar changed to dark (d) or light (l) orange. – 0.0 (no colour change); ± 0.1- 0.2; + 0.3-1.0; ++ 1.1-2.0 and +++ 2.0-4.0 mm/day for the advancement of the colour change front in the CAS-blue agar.

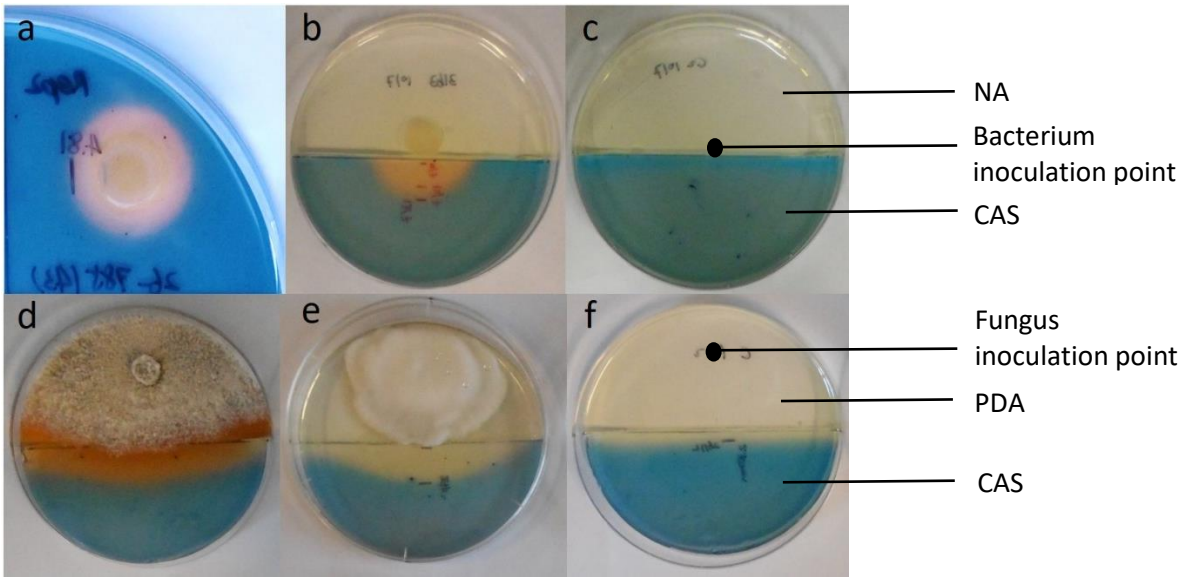

Figure 3.9 Chrome Azurol S (CAS) and modified CAS agar (MCAS) plate assays for testing siderophore production by endophytic bacteria and fungi. a) Endophytic bacterial isolate 26-785(43)b producing orange halo in the CAS assay. b) Endophytic bacterial isolate 31b3 producing orange colour in the MCAS assay (bottom half: CAS-blue agar, top half: NA). d) and e) Endophytic fungal isolates 2-66f and 6-176f producing orange colour in the MCAS assay, respectively (bottom half: CAS-blue agar, top half: PDA). c) and f) Control plates in the MCAS assay for bacteria and fungi, respectively.

3.3.9 Evaluation of volatile compound production by the selected endophytic bacteria and fungi

Volatiles produced by endophytic bacterial isolates 31b1, 27-801(89)b, R3L-6b, 21-606(28)b, 20-579(18)b, 26-771(23)b, 42-1206(19)b and R1GS-12b significantly promoted the growth of *N. ditissima* ICMP14417 compared with the control (Table 3.17; Appendix A3.17). For endophytic fungi, volatiles produced by isolate 3-73f significantly increased the growth *N. ditissima* ICMP14417 compared to the control (Table 3.17; Appendix A3.18). Volatiles produced by the remaining tested endophytic bacterial and fungal isolates did not affect the radial growth of *N. ditissima* ICMP14417 compared with their respective control (Table 3.17).

Table 3.17 The effect of volatile production from selected bacterial and fungal isolates on radial growth of *Neonectria ditissima* ICMP14417.

Bacterial isolate	Radial growth (mm)	Fungal isolate	Radial growth (mm)
Control	23.4 fgh	Control	18.0 bcdef
Pseudomonas sp. 31b1	27.9 a	*Neosetophoma* sp. 3-73f(S)	19.5 a
Pseudomonas sp. 27-801(89)b	27.8 ab	*Chaetomium* sp. 1-35f (F)	19.5 ab
Bacillus sp. R3L-6b	27.5 ab	*Epicoccum* sp. 35-1049(2)f(M)	19.1 abc
Bacillus sp. 21-606(28)b	27.2 abc	*Biscogniauxia* sp. 10-283(1)f (F)	19.1 abcd
Pseudomonas sp. 20-579(18)b	26.0 bcd	*Biscogniauxia* sp. 2-66f(F)	19.0 abcd
Bacillus sp. 26-771(23)b	25.7 cd	*Epicoccum* sp. 34-1029(1)f(M)	18.9 abcd
Bacillus sp. 42-1206(19)b	25.2 de	*Chaetomium* sp. 2-57f(F)	18.7 abcdef
Bacillus sp. R1GS-12b	25.2 de	*Epicoccum* sp. 36-1073(1)f(M)	18.6 abcde
Pseudomonas sp. 7-208(18)b	25.1 def	*Biscogniauxia* sp. 20-594f(F)	18.1 abcdef
Pseudomonas sp. 31b3	24.8 def	*Epicoccum* sp. 34-1026(3)f(M)	17.9 bcdef
Bacillus sp. R3L-1b	24.4 defg	*Epicoccum* sp. 4-105(2)f(M)	17.8 cdef
Bacillus sp. 41-1183(7)b	24.4 defg	Unidentified 20-578f(F)	17.6 def
Pseudomonas sp. 26-785(43)b	23.7 efgh	*Biscogniauxia* sp. 2-51f(F)	17.1 ef
Pseudomonas sp. 39-1143(30)b	22.7 gh	*Chaetomium* sp. 1-38f(F)	16.9 f
Bacillus sp. 41-1183(6)b	22.7 gh	*Penicillium* sp. 6-176f(M)	-
Bacillus sp. 41-1182(4)b	22.5 h		
p value	< 0.001	*p* value	0.037

The letters after radial growth (mm) are based on the results of the pairwise comparisons using one-way ANOVA in Minitab 17. Mean values followed by the same letter are not significantly different. – means no data as isolate 6-176f(M) grew across to the compartment containing *N. ditissima* ICMP14417. F, M and S in the bracket represent fast-growing fungi, medium-growing fungi and slow-growing fungi, respectively.

3.3.10 Detection of antibiotic producing genes

3.3.10.1 Detection of *bamC*, *fenD*, *sfp* and *ituD* in *Bacillus* spp. isolates by PCR

PCR detection and sequencing results confirmed *bamC* was present in isolates R1GS-12b and 42-1206(19)b. After PCR cycles were increased to 35, bands similar to the target size were also obtained from isolates 21-606(28)b, 26-771(23)b, 41-1183(6)b, 41-1183(7)b and R3L-1b. Only the PCR product of 41-1183(7)b was sequenced and showed weak similarity to bacillomycin D (81% to AHW47908.1). Therefore, the bands amplified from isolates 21-606(28)b, 26-771(23)b, 41-1183(6)b, 41-1183(7)b and R3L-1b were considered as unidentified. *FenD* was not detected by PCR in any of the nine *Bacillus* spp. isolates. The *sfp* gene was only confirmed as present in isolate R3L-1b. *ItuD* was confirmed in isolates R1GS-12b and 42-1206(19)b. None of the *Pseudomonas* spp. isolates were positive for any of the four genes *bamC*, *fenD*, *sfp* and *ituD*. The encoding genes identified as positive by PCR agarose gel and/or sequencing are shown in Appendix A3.19.

3.3.10.2 Detection of *phlD*, *phzC*, *prnC*, *pltC* and *hcnBC* in *Pseudomonas* spp. isolates by PCR

phlD, *phzC*, *prnC*, *pltC* and *hcnBC* were not detected by PCR in any of the seven *Pseudomonas* spp. or *Bacillus* spp. (data not shown).

3.4 Discussion

Antagonistic endophyte community obtained

This is the first New Zealand study to investigate the ability of culturable microbial endophytes from apple to inhibit the *in vitro* growth of *N. ditissima*, the causal agent of European canker. Of 1004 bacteria and 87 fungal endophytes isolated from apple shoot tissues, 18 bacteria and 17 fungi were able to inhibit *N. ditissima* mycelial growth. From those isolates, 16 bacterial and 14 fungal isolates were further selected based on their rDNA sequence similarity to reported endophytes and/or known biocontrol agents, but not plant pathogens. *In vitro* biocontrol functional assays were used to extend our understanding of their biocontrol potential and mode of action in controlling *N. ditissima*.

A relatively low frequency of endophytic bacteria with biocontrol ability were isolated in this study (18 out of 1004 bacterial isolates), when compared to recent studies of endophytes recovered from domesticated and wild plants. For example, Wicaksono et al. (2016) showed that 33 and 39 isolates out of 192 endophytic bacteria recovered from the New Zealand native medicinal plant mānuka (*Leptospermum scoparium*) inhibited colony growth of *Ilyonectria liriodendri* and *Neofusicoccum luteum*, respectively. In their work on apple, Miliute et al. (2016) recovered 38 endophytic bacteria from apple buds of different cultivars and identified three

isolates that inhibited the *in vitro* growth of the apple scab pathogen *Venturia inaequalis*. The low frequency of antagonistic bacterial isolates identified in this study could be because only apple leaf and stem were used for isolation, resulting in a lower diversity of bacteria. Wicaksono et al. (2016) reported the greatest proportion of bacterial isolates antagonistic to *N. luteum* were recovered from root tissue samples of mānuka (n = 31), followed by stem samples (n = 8), but none from leaf tissue. They also found the bacterial isolates with the strongest inhibition to *I. liriodendri* were recovered from roots of mānuka. This study also obtained relatively higher number of bacterial isolates with antagonism to *N. ditissima* (≥ 20% inhibition effect) from stem (n = 11) than from leaf (n = 7) (Appendix A3.9).

As compared to endophytic bacteria, more culturable fungal endophytes (17 out of 87 endophytic fungal isolates representing 33 morphotypes) with *in vitro* antagonism to *N. ditissima* were identified. This is because a representative number of fungal isolates were selected based on their colony morphology diversity, and then tested for their *in vitro* inhibition effect on *N. ditissima*. This could not be done for bacteria due to the lack of morphological features for selection. A similar study conducted by Mejía et al. (2008) found 21 out of 52, 28 out of 43, and 4 out of 15 endophytic morpho-species recovered from *Theobroma cacao* had *in vitro* antagonism against three fungal pathogens of cacao, *Moniliophthora roreri* (frosty pod rot), *Phytophthora palmivora* (black pod rot) and *Moniliophthora perniciosa* (witches broom), respectively.

The recovered collection was only representative of the endophytic bacteria and fungi. Representative isolates were selected from isolation plates using a random number selection method and based on fungal morphology diversity, respectively, providing good representation of the culturable endophyte community within the apple tissues. A potential issue with this approach could be the single agar type used for isolation, which may have biased the diversity of bacterial/fungal taxa isolated and therefore the diversity of bacteria/fungi tested. Using a greater variety of culture media could improve the diversity of recovered isolates (Zheng et al., 2016). Also, fast-growing and sporulating bacteria and fungi may overgrow slower-growing isolates. However, the low nutrient isolation agars (R2A for bacteria and SNA for fungi) used in this study are designed to allow recovery of slow growing micro-organisms (Mclnroy & Kloepper, 1995; Zheng et al., 2016). Mclnroy and Kloepper (1995) also found R2A was the best isolation agar as compared to tryptic soy agar (TSA) and medium SC because there was less colony overlap, smaller colony size and it allowed for the growth of the same heterotrophic bacteria.

The single actinobacterium recovered in this study (from the leaf of 'Royal Gala') indicated a low frequency of culturable taxa in apple leaves and stems despite the DGGE indicating that there were several species present in apple leaves and stems (Chapter 2). This was less than expected

given the nine isolated by Purushotham et al. (2018) from horopito (*Pseudowintera colorata*), 10 from healthy roots and stems of the medicinal plant *Aquilaria crassna* (Nimnoi et al., 2010) and 576 from leaves, stems and roots of two *Eucalyptus* species, a native apricot (*Pittosporum phylliraeoides*) and a native pine (*Callitris preissii*) (Kaewkla & Franco, 2013). Previous studies have also demonstrated recovery of actinobacteria from leaves, stems and roots of different plants (Kaewkla & Franco, 2013; Qin et al., 2012; Qin et al., 2015). A possible reason for the lower recovery frequency is that domesticated plant species may have lower diversity than the native and medicinal plant species used in the examples of prior work, although there is no literature reporting comparisons between domesticated and native/medicinal plants. Pérez-Jaramillo et al. (2016) have suggested that domestication of plants reduces their genetic diversity compared to their wild ancestors, and that this also affects microbial diversity through decreased complexity of plant-microbe interactions. Other reasons may include the single agar type or limited number of plant tissues used for isolation. Others have shown that to study actinobacteria, three to five media with different components can be required (Jiang et al., 2016). Although time prohibited this in the current study, use of more media types may enhance recovery. This would be expected to increase the isolation of potential biocontrol candidates as actinobacteria were reported as important antibiotics producers (Palaniyandi et al., 2013). This is the first study to isolate an actinobacterium endophyte from apple leaves and stems. As the recovered actinobacterium did not inhibit the growth of *N. ditissima* in the dual culture plating assay, it was excluded from the further study.

In the current study, only bacterial isolates with antagonistic activity against *N. ditissima* colony growth were taxonomically identified. Therefore, the overall diversity of the culturable bacterial endophyte community is unknown. Other studies suggest that apple may host several bacterial genera. Muresan (2017) recovered 55 endophytic bacterial isolates belonging to 12 genera from a wide range of apple tissue types, geographic locations, apple varieties and management type. Similar to the current study they also found *Bacillus* species were the most effective isolates for *in vitro* inhibition, in that case of *Venturia inaequalis* causing apple scab. However, the overall diversity of culturable fungal community was determined based on their morphotypes.

Based on fungal morphotypes identified in this study, the culturable fungal community was un-affected by factors including regions, sites, varieties, seasons, management practices and infection levels investigated in this work. However, the effect of those factors on the culturable fungal community may have been underestimated due to the unbalanced sampling conducted in this work. For instance, sample size of commercial varieties was much smaller than heritage varieties (4 commercial varieties vs. 35 heritage varieties) due to the limited varieties available in

the HBHV site. Unbalanced sampling also occurred in the spring (M1) and autumn (M2) samplings for investigating season, management practice and infection levels. The unbalanced sample size could have biased diversity of morphotypes from samples associated with different factors (Germida et al., 1998; Lilley et al., 1996). Different representative number of fungal isolates selected from the SNA isolation plates in different samplings (M1 = 25, M2 = 10 and HBHV = 10) was not the reason for the potential underestimation, since isolates with different colony morphologies were able to be selected from the different samplings. From the culturable fungal community obtained in this work, endophytic fungal isolates with antagonism to *N. ditissima* were identified.

Antagonistic isolates recovery frequency affected by orchard factors

This study demonstrated that season affected the proportion of bacteria with *in vitro* biocontrol activity against *N. ditissima*. More antagonistic bacteria were recovered from the main sampling in autumn (9%, 5 out of 58 tested isolates) than the main sampling in spring (1%, 8 out of 725 tested isolates), with a higher proportion of antagonistic *Bacillus* spp. obtained in autumn (4 out of 5 isolates) and a higher proportion of antagonistic *Pseudomonas* spp. obtained in spring (6 out of 8 isolates). The higher percentage of antagonistic bacterial isolates obtained in autumn may be due to the higher proportion of *Bacillus* spp. which were more antagonistic against *N. ditissima in vitro* than *Pseudomonas* spp. Also, all three antagonistic bacterial isolates recovered from the LU orchard sampling conducted in autumn were *Bacillus* spp. This could be associated with the changing physiology of the plant due to seasonal effects. Malaguti et al. (2001) reported that amino acid concentrations in apple xylem sap increased from spring when buds burst, peaked at full bloom and then reduced during fruiting. This suggests that lower nutrients are available in autumn and maybe antagonistic *Bacillus* isolates are better able to colonise under this condition. However, care must be taken in any comparison between autumn and spring samplings in the study due to the large differences in the bacterial isolate sample size, with 58 isolates for autumn and 725 isolates for spring. Sample size determined the number of bacterial taxa obtained and thereby affected the number of genera identified with specific characteristics (Germida et al., 1998; Lilley et al., 1996) such as being antagonistic to *N. ditissima*. Further work to increase the autumn sample size could confirm the effect of season on the frequency of antagonistic bacterial endophytes in apple tissue. Thereby, potential antagonistic endophytic species prevalent in all seasons could be identified and these may be more durable in their ability to colonise apple tissue and protect against *N. ditissima* infection all year round. The ability of the isolates to colonise apple tissue in both spring and autumn will be determined in Chapter 4.

The higher percentage of antagonistic bacteria recovered from 'Royal Gala' (14%, 4 out of 29 tested isolates) than 'Braeburn' (3%, 1 out of 29 tested isolates) in the M2 sampling (autumn) may indicate the frequency of culturable bacteria antagonistic to *N. ditissima* was affected by apple variety. Plant variety affected the isolation of antagonistic endophytic bacteria in Betelvine (*Piperaceae*) with more recovered from the wild-type variety than the cultivated variety (Singh et al., 2017). Different endophyte recovery frequency in different cultivars could be associated with the unique niches provided by the different cultivars. Endophyte colonisation in sugarcane was dependent on the xylem structure which differed between varieties (Dong et al., 1997). Therefore, plants may work as filters, selectively recruiting endophytes (Etesami et al., 2014). However, care must be taken in making any conclusion regarding differences in the population of endophytic bacteria antagonistic to *N. ditissima* between 'Royal Gala' and 'Braeburn' in the current study due to the small sample size of only 29 isolates for each variety in the autumn sampling. Frequency of antagonistic bacterial isolates in the M1 sampling (spring) is unlikely to provide reliable information for evaluating the effect of apple variety because the frequency of antagonistic bacterial isolates in this sampling was low for both 'Royal Gala' and 'Braeburn'. Future studies should be carried out with increased number of samples and cultivars sampled to investigate the effect of apple cultivar on endophytic bacteria with activity against *N. ditissima*.

Biocontrol potential identification

The criterion for selection of antagonistic endophytic bacterial and fungal isolates for further study was based on their genetic similarity to known candidate biocontrol agents. Thirteen bacterial isolates showed high sequence similarity to species with biocontrol activity, including *B. cereus*, *B. thuringiensis*, *B. toyonensis*, *B. subtilis*, *B. amyloliquefaciens*, *P. fluorescens*, *P. azotoformans*, *P. brenneri*, *P. orientalis* and *P. poae*. All these species have reported biocontrol ability, with some commercialised and registered for use (Fang et al., 2016; Ferreira et al., 1991; Handelsman et al., 1990; Howell & Stipanovic, 1980; Kurniawan et al., 2018; Lopes et al., 2017; Magnin-Robert et al., 2007; Reyes-Ramírez et al., 2004; Vidhyasekaran & Muthamilan, 1995; Zachow et al., 2015; Zengerer et al., 2018). *Bacillus amyloliquefaciens* strains are used in biological control products such as 'TripleX' (biostart.co.nz) and 'BacStar' (etec.co.nz) which are registered in New Zealand for the control of Botrytis in grapes and other crops, and Botrytis and powdery mildew of a range of crops, respectively. Three isolates showed similarity to *B. horneckiae* and/or *B. foraminis*, and although there was no information regarding their activity as biocontrol agents they were still included for further study. Although the results showed that several isolates with activity against *N. ditissima* were reported to be potential biocontrol agents, identification was done using only a partial 16S RNA gene sequence. Sequencing of more genes,

such as the housekeeping genes *gyrB* and *rpoD,* would clarify identity to species or strain level (Everett et al., 2011; Sarkar & Guttman, 2004; Wang et al., 2007).

Isolates from six genera, obtained from apple leaves and stems, were shown to inhibit the *in vitro* growth of *N. ditissima*, revealing diverse endophytic fungi with potential to control European canker. The six genera are *Biscogniauxia*, *Diaporthe*, *Chaetomium*, *Epicoccum*, *Phylctema* and *Penicillium*, species of which have been previously isolated from apple tissues, except *Chaetomium*. *Epicoccum* and *Penicillium* species have been reported as endophytes recovered from apple tissues (Camatti-Sartori et al., 2005; Muresan, 2017). Species of *Biscogniauxia* (eg. *B. marginata*), *Diaporthe* (*D. tanakae*) and *Phylctema* (*P. vagabunda*) have been reported as apple pathogens by Amiri et al. (2008), Henriquez (2005) and the American Phytopathological Society (https://www.apsnet.org/publications/commonnames/Pages/Apple.aspx). These were excluded from further study, except isolates showing high similarity to *Biscogniauxia* sp. 1 ICMP 18828 or *Biscogniauxia* sp. 2 ICMP 18793 which were both identified as *B. kuntze* and this species has been reported as endophytes of *Nothofagus* species in New Zealand (Johnston et al., 2012). This is the first report of *Chaetomium* sp., a well-documented fungal group used as biocontrol agents (Soytong et al., 2001), as endophytes from apple. In addition, isolate 3-73f, identified as belonging to the order Pleosporales (*Neosetophoma* sp./*Leptosphaeria* sp./*Coniothyrium* sp) required further sequencing in order to confirm identity.

Thirteen endophytic fungal isolates belonging to the genera *Chaetomium* (n = 3), *Epicoccum* (n = 5), *Biscogniauxia* (n = 3), *Penicillium* (n = 1) and *Neosetophoma* sp./*Leptosphaeria* sp./*Coniothyrium* sp. (n = 1) were selected as the most promising candidates for suppressing *N. ditissima*, due to their sequence similarity to known biocontrol agents. For example, *Chaetomium globosum* has been developed as a broad-spectrum biological fungicide for controlling a wide range of fungal pathogens in different plants such as *Phytophthora palmivora* in black pepper, *Fusarium oxysporum* in tomato and *V. inaequalis* in apple (Cullen et al., 1984; Soytong et al., 2001). *Epicoccum nigrum* has been widely studied as a source of biocontrol agents against various fungal pathogens in plants (Hashem & Ali, 2004; Kortekamp, 2015; Larena et al., 2005). *Biscogniauxia* species and *P. ochrochloron* produce antimicrobial metabolites and have potential antimicrobial activity (Carvalho et al., 2016; Patil et al., 2013; Rančić et al., 2006; Silva-Hughes et al., 2015). *Leptosphaeria rubefaciens* and *Coniothyrium* sp. are endophytes of ash (*Fraxinus excelsior*) (Haňáčková et al., 2017; Kosawang et al., 2018), with a *Coniothyrium* sp. found to have *in vitro* antagonism against *Hymenoscyphus fraxineus* causing ash dieback disease (Haňáčková et al., 2017). There are no reports in the literature of either *Neosetophoma clematidis* or *N. italica* being plant pathogens, but species of this genus are endophytes of *Fraxinus* spp. (Kosawang et al.,

2018). As fungal isolates selected in this study were only identified to genus level, further gene sequences are needed to confirm their identity. The combined use of the ITS region with sequences of other gene regions such as the *β-tubulin*, *tef1α*, RPB1 or RPB2 would enable more definitive identification of fungal isolates to species level (Bogner et al., 2016). However, the current identification level was sufficient for selection of promising antagonistic fungal isolates against *N. ditissima*. Further work is required to determine their potential for suppressing European canker.

The majority (15 of the 16 isolates) of the selected bacterial isolates produced inhibitory compounds in the absence of the pathogen *N. ditissima* ICMP14417 and these accumulated over time, indicating secretion of inhibitory metabolites was constitutive. Using the same method as this study, Purushotham Balraj (2017) found all the bioactive bacterial strains isolated from horopito secreted bioactive metabolites constitutively. However, results showed that the secretion of inhibitory metabolites by *Pseudomonas* isolate 31b1 was induced by the presence of the pathogen. This could be because secondary metabolites such as antibiotics produced by isolate 31b1 required a biosynthesis inducer and/or growth rate decrease caused by the presence of *N. dittisima* (Demain, 1998). However, further studies are required to confirm this and the mechanisms involved. As the inhibitory compounds could be produced by the test bacterial isolates without the presence of the pathogen, the inhibitory effect of the cell-free filtrate was further investigated.

Only the cell-free culture filtrates from two *Bacillus* isolates (42-1206(19)b and R1GS-12b) and one *Pseudomonas* isolate (27-801(89)b) significantly inhibited the radial growth of *N. ditissima*, indicating production of antifungal compounds which inhibited mycelial growth by the pathogen. Fewer bacterial isolates could inhibit *N. ditissima* when the agar was amended with cell-free culture filtrate compared with live bacterial isolates paired against the pathogen and this was also reported by Kurniawan et al. (2018). This could be due to the concentration of antifungal compounds in the culture filtrates of the remaining 13 isolates being low. In the current study the bacterial isolates were grown in the Waksman broth for 16 h and 38 h prior to the culture filtrate being used, whereas in the dual culture assay the bacterial isolates grew for 17 days on the Waksman agar which may have allowed higher concentrations of any diffusible antifungal compounds to accumulate. Romero et al. (2007) reported that the antifungal activity of *B. subtilis* strains liquid cultures was highest after 4 to 5 days of growth. Chaiharn et al. (2009) reported production of siderophores, which are important secondary metabolites contributing to biocontrol properties, was affected by incubation times and temperatures, with maximum production by *Pseudomonas aureofaciens* AR 1 liquid culture after incubation for 15 days at 30°C.

Media type could also affect the inhibitory ability of the candidate isolates with Bonsall et al. (1997) reporting that *Pseudomonas fluorescens* Q2-87 produced 47.7 times greater concentrations of the antibiotic 2,4-DAPG on solid YM medium than in liquid medium. Moreover, inhibition of *N. ditissima* colony growth was only seen with amendment with relatively high concentrations of culture filtrate (10% or 30%), indicating low concentrations of the inhibitory compounds in the culture filtrate. Amending the Waksman agar with higher concentrations of culture filtrates may have improve the inhibition effect for the 13 isolates which did not show significant bioactive effect in both tested concentrations. This could be achieved by concentrating the culture filtrates (Leunk et al., 1988).

The slow growth of three of the *Bacillus* spp. isolates (41-1182(4)b, 41-1183(6)b and 41-1183(7)b) during the 38 h incubation period for liquid culture indicated growth rate difference among *Bacillus* species/strains. The possible reason could be different growth requirements of varied *Bacillus* species. The three isolates were in the same cluster in the NJ trees (Section 3.3.4.1) indicating that they are genetically similar and therefore more likely to have similar growth requirements (Goldberg et al., 1994). Incubation temperature and aeration condition could be modified for accelerating their growth rate (Andersen & von Meyenburg, 1980; Nedwell, 1999).

The cell-free filtrate of *Chaetomium* sp. 1-38f, *Epicoccum* sp. 34-1029(1)f and *Phlyctema* sp. 36-1072f produced diffusible antifungal compounds able to reduce mycelia growth of *N. ditissima*. Both *Epicoccum nigrum* and *Chaetomium globosum* are known to produce antifungal compounds in liquid culture, such as flavipin produced by *E. nigrum*, and chaetomin and chaetoviridins produced by *C. globosum* (de Lima Favaro et al., 2012; Di Pietro et al., 1992; Madrigal et al., 1991; Park et al., 2005). Culture filtrates from the other two *Epicoccum* sp. isolates (34-1026(3)f and 35-1049(2)f) did not inhibit *N. ditissima* colony growth indicating strain or species variation. Overall, none of the cell-free culture filtrates from the fungal isolates tested strongly inhibited *N. ditissima* colony growth when incorporated into the growing media which, as discussed for the bacterial isolates, could be due to a low concentration of antifungal compounds in the liquid culture. As discussed previously, collection and testing of culture filtrates collected after different incubation times could be investigated to see if there was an increase in ability to inhibit *N. ditissima*.

The modified CAS (MCAS) method allowed siderophore production by all 16 selected endophytic bacteria to be assessed. This method was particularly useful as it enabled siderophore production by the gram-positive *Bacillus* species, which did not grow on the CAS medium, to be evaluated. Most isolates (15 of the 16 isolates) produced siderophores (Sid^{+}), including *Bacillus* spp. and *Pseudomonas* spp. isolates. Other studies have reported that strains of *Pseudomonas* and *Bacillus* species can produce siderophores (Matthijs et al., 2007; Pal et al., 2001; Yu et al., 2011).

Purushotham Balraj (2017) found 66% of actinobacterial endophytes isolates did not produce siderophores but he did not use the modified CAS method and did not state how many isolates failed to grow on the CAS media. Similarly, Wicaksono et al. (2016) reported 58% of bacterial endophytes isolates from mānuka were positive for siderophore production, but they also did not state how many isolates failed to grow on the CAS media. However, only bacterial isolates with *in vitro* inhibition effect on *N. ditissima* were tested for siderophore production. This could be another reason for a higher percentage of Sid^+ isolates in the current study.

In general, *Pseudomonas* spp. had higher siderophore activity than *Bacillus* spp., with *Pseudomonas* isolates 31b1, 31b3 and 27-801(89)b having the greatest siderophore production ability. This may indicate that siderophore production was the main biocontrol mechanism for the selected *Pseudomonas* isolates in the dual culture plate assays. Chaiharn et al. (2009) also reported that *P. aureofaciens* AR 1 showed higher siderophore production, as compared to isolates with antifungal activity from the other genera including *Bacillus*, *Kocuria*, *Streptomyces* and *Ochrobactrum*. Greater siderophore activity is due to the isolate either producing more siderophores and/or the siderophores produced having higher affinity for chelating iron (Loaces et al., 2011). However, this was not investigated in the current study.

All the fungal species identified as potential biocontrol candidates in this study produced siderophores, except for *Chaetomium* sp. 2-57f and *Biscogniauxia* sp. 2-51f. Of the species identified as producing siderophores, only *Penicillium* (eg. *Penicillium citrinum* VFI-51) and *Chaetomium* isolates have previously been shown to have this capability, which has been shown to contribute to their antifungal activity or plant growth enhancement ability (Haruma et al., 2018; Sreevidya et al., 2015). This is the first study to show that *Biscogniauxia* spp. and *Epicoccum* spp. produce siderophores which may contribute to their potential in inhibiting plant pathogens, but this would need to be confirmed.

In the current study, *Bacillus* species (n = 9) had comparatively greater *in vitro* biocontrol activity against *N. ditissima* (ICMP14417, MW15c1 and RS324p) than the *Pseudomonas* species (n = 7) in the dual plate culture. Similarly, Karimi et al. (2012) found that *B. subtilis* strains (n = 6) were better able to inhibit growth of the fungal pathogen *Fusarium oxysporum* f. sp. *ciceris* than *Pseudomonas* isolates. A potential reason is that metabolites produced by the *Bacillus* species are more inhibitory towards *N. ditissima in vitro* than those produced by *Pseudomonas* species. Bacterial species are known to have antagonistic activity against different fungal pathogens based on the bioactive metabolites produced. Karimi et al. (2012) reported that *B. subtilis* strains produced more non-volatile inhibitory metabolites, which diffused in the agar, whilst *Pseudomonas* isolates produced more volatile compounds. This could be the reason for *Bacillus*

spp. being more inhibitory towards *N. ditissima* as non-volatile metabolites would not evaporate during the incubation period. However, this needs to be confirmed by modified CF filtrate assay as previously discussed.

In the volatile assay none of the 16 selected bacterial isolates or 14 tested fungal isolates inhibited the growth of *N. ditissima*, indicating they did not produce bioactive volatile compounds with antagonism effect under the experimental conditions used in the study. This is in contrast to the literature which shows that *Pseudomonas* isolates can produce volatile compounds inhibitory to the growth of *Fusarium oxysporum* f. sp. *ciceris* (Karimi et al., 2012). In addition, *Epicoccum nigrum* (Lahlali & Hijri, 2010) and *Biscogniauxia* spp. (Ulloa-Benítez et al., 2016) have been reported to produce inhibitory volatile compounds contributing to their biocontrol activity. The lack of detection of inhibitory volatile production could also be because of the complexity of the volatile compound mixture which may include components exhibiting positive, negative or neutral interactions with other microorganisms (Wheatley, 2002).

Two *Bacillus* isolates R1GS-12b and 42-1206(19)b were likely to produce bacillomycin D (encoded by *Bam C*) and iturin A (encoded by *ItuD*), and *Bacillus* isolate R3L-1b to produce surfactin (encoded by *sfp*), indicating they had the potential to suppress plant pathogens by synthesising antibiotics. Bacillomycin D and iturin A belonging to the iturin family of lipopeptides, and surfactin have all been reported to play an important role in the antifungal activity of strains of *B. subtilis* and *B. amyloliquefaciens* (Chen et al., 2009; Kim et al., 2010; Peypoux et al., 1984; Xu et al., 2013; Yu et al., 2002). This is consistent with these three *Bacillus* isolates having high sequence similarity to *B. subtilis* or *B. amyloliquefaciens*. The gene encoding the production of Fengycin (*fenD*), also an important lipopeptide-type antibiotic (Chen et al., 2009; Chen et al., 2007; Ongena et al., 2005; Romero et al., 2007), was not found in any of the *Bacillus* sp. isolates in this work. Further, none of the four antibiotics encoding genes were detected in most of the test *Bacillus* isolates, indicating that other antifungal metabolites were involved in the inhibition of *N. ditissima* growth by these isolates. Additional genes such as the gene cluster including *fenF*, *mycA*, *mycB* and *mycC* involved in mycosubtilin synthesis, another important lipopeptide antibiotics with antifungal activity, could be targeted for detection (Leclère et al., 2005). Moreover, additional antibiotic encoding genes known to be present in other *Bacillus* spp. identified in this work could be targeted, such as the gene cluster related to zwittermicin A biosynthesis in *B. cereus* and *B. thuringiensis* (Kevany et al., 2009; Shao et al., 2008; Zhao et al., 2007). However, the two *Bacillus* isolates showing the presence of baciliomycin and iturin A encoding genes were also seen to be the most effective isolates at inhibiting *N. ditissima* growth, with over 80% inhibition against all three *N. ditissima* isolates.

None of the *Pseudomonas* spp. isolates contained genomic sequences with homology to *phlD* (encoding 2,4-diacetylphloroglucinol), *phzC* (encoding phenazine), *prnC* (encoding pyrrolnitrin), *pltC* (encoding pyoluteorin) and *hcnBC* (encoding hydrogen cyanide), demonstrating that these antibiotics were not involved in the inhibition of *N. ditissima* growth by these isolates. However, these five antibiotics are well known mechanisms for the biological control of diseases by *Pseudomonas* spp., especially for fluorescent *Pseudomonas* spp., the most important group of *Pseudomonas* spp. for suppressing soilborne fungal pathogens (Kirner et al., 1998; Mavrodi et al., 2001; Mazurier et al., 2009; Paulin et al., 2009; Raaijmakers et al., 2002; Ramette et al., 2003). It is possible that fluorescent *Pseudomonas* spp. with antifungal activity against trunk pathogens *N. ditissima* may have a different suite of antibiotics. However, there is limited information regarding other antibiotic encoding genes present in *Pseudomonas* spp. For example, only candidate gene clusters involved in secondary metabolite production were found for *P. azotoformans* (Fang et al., 2016). Most of the *Pseudomonas* isolates selected in this work were identified to be strongly similar to *P. fluorescens*. Therefore, the failure in obtaining PCR products for the five target genes from *Pseudomonas* isolates, as well as the four target genes from *Bacillus* isolates maybe also because of low copy numbers or base substitutions in the target sequences (Kim et al., 2013).

A number of bacteria (n = 16) and fungi (n = 13) were identified as biocontrol candidates to inhibit *in vitro* growth of *N. ditissima*. Further studies are required to confirm the biocontrol properties especially antibiotics and siderophore production ability by selected isolates, in order to enable the *in vitro and in vivo* biocontrol activity of the isolates to be enhanced. In the next Chapter, the colonisation ability of the selected bacterial isolates were investigated which acted as another criteria for selecting potential biocontrol agents for suppressing European canker *in planta*.

Chapter 4 Colonisation and persistence of selected antagonistic endophytic bacteria in detached apple shoots

4.1 Introduction

Neonectria ditissima is a trunk pathogen that requires wounds to penetrate plants, such as leaf scars and mechanical wounds produced by weather damage, pruning or harvesting (Alves & Nunes, 2017; Ghasemkhani, 2012; Ohlendorf, 1999; Weber, 2014). Conidia of *N. ditissima* are produced in spring and dispersed by rain to infect new woody tissues from sources up to three metres from the tree and one metre up the tree canopy (Anon., No date-c). During summer and autumn, perithecia are produced on old cankers when the weather is wet, with ascospores released from mature perithecia after rainfall. The released ascospores are wind dispersed at distances from hundreds of metres to even a few kilometres to cause new infections (Agrios, 1997). Thus, for effective and long-term control of *N. ditissima* protection of wounds from infection by these spores is required. For endophytic bacteria to provide effective control of this pathogen they must be able to colonise wounds of apple woody tissues after inoculation and move inside the stem tissues to provide a durable barrier to the near year round pathogen attack.

Endophytic bacteria with *in vitro* antimicrobial activity isolated from *Leptospermum scoparium* (mānuka) showed *in vivo* colonisation in grapevines and kiwifruit (Wicaksono, 2016). Similar results were found by Chen et al. (1995) in cotton. The 16 antagonistic endophytic bacterial isolates showing *in vitro* inhibition of *N. ditissima* in Chapter 3 were selected as candidates for assessment of colonisation in detached apple shoots. Colonisation by inoculated endophytes can be tracked by selective isolation from inoculated shoots, however this requires the bacterium to be easily differentiated from others resident in the detached material. The use of genetically modified (GMO) inoculant bacteria can be an effective way to trace endophytic colonisation in *planta* (Amarger, 2002). However, the use of GMO's is highly regulated in New Zealand and they cannot be introduced into field ecosystems. In contrast, spontaneous antibiotic resistance mutants are not considered to be GMO's and their use is less regulated. Resistance to antibiotics in bacteria is caused by mutations, such as the mutations in the *rpoB* gene responsible for rifampicin resistance (Morlock et al., 2000). Therefore, there is still a risk of possible transfer of resistance genes to unwanted organisms like pathogens (Macheras et al., 2011), so care must be taken when selecting the antibiotic to minimise this risk. Spontaneous antibiotic resistant mutants were successfully applied by Wicaksono et al. (2018) to monitor endophytic colonisation of antagonistic bacteria in kiwifruit vines and will therefore be used in the current chapter.

The overall aim of this chapter was to determine the ability of candidate endophytic bacteria to colonise wound sites and to persist at the site of introduction in detached apple shoots. It included three specific objectives which were (i) to produce antibiotic resistant bacterial mutants, (ii) to verify *in vitro* biocontrol activity of the bacterial mutants as compared with wild types and, (iii) to determine the colonisation ability of the bacterial mutants in apple stems.

4.2 Materials and methods

4.2.1 Selection of antibiotics for producing endophytic bacterial mutants

To enable the colonisation of apple stems by the endophytic bacteria to be assessed, attempts were made to produce spontaneous antibiotic-resistant mutants of each of the selected bacteria. This enabled the selective isolation of the inoculated bacterium from apple stems onto antibiotic amended agar.

In order to select the antibiotic(s) for producing endophytic bacterial mutants, the antibiotic which most successfully inhibited the growth of the background endophytes found in apple stems was determined. Apple stem segments from the middle part of one-year old apple shoots (approx. 5 cm long) were collected from the Lincoln University Research Orchard and surface sterilised as previously described (Section 2.2.2). Then, they were cut horizontally into pieces (approx. 1 mm thick) and plated on nutrient agar (NA) containing streptomycin (Sigma, Sigma-Aldrich, USA), rifampicin (Sigma, Sigma-Aldrich, USA; and PhytoTechnology Laboratories, USA), chloramphenicol (Sigma, Sigma-Aldrich, USA) or erythromycin (Sigma, Sigma-Aldrich, USA) at concentrations of 50 ppm (μg/mL), 75 ppm and 100 ppm. Three replicate plates were set up for each concentration of each antibiotic, with six stem pieces on each plate. All the plates were incubated at 25°C in darkness for 8 days. The antibiotic(s) which inhibited the growth of background endophytic bacteria from apple stems at the lowest concentration were selected.

The mean number of stem pieces with growth of bacteria on the NA amended with each concentration of each antibiotic were calculated and analysed with one-way ANOVA to determine the significance of the antibiotic treatments followed by Fisher's protected LSD test at $p \leq 0.05$ using Minitab 17 (Lead Technologies, Australia).

4.2.2 Production of spontaneous antibiotic-resistant endophytic bacterial mutants

Attempts were made to produce spontaneous antibiotic-resistant mutants for all 16 endophytic bacterial isolates selected from the results of Chapter 3 using the antibiotics shown to reduce the growth of background apple endophytic bacteria in Section 4.2.1. The bacterial isolates were successively subcultured on NA amended with increasing concentrations of the corresponding

antibiotic at 10, 20, 50, 75, 100 and 125 ppm. For each endophyte bacterial isolate, a bacterial mutant colony with the highest resistance to the antibiotic was selected, subcultured into nutrient broth (NB) and stored at -80°C in 20% glycerol (LabServ, Thermo Fisher Scientific, New Zealand).

4.2.3 Stability assays of the spontaneous antibiotic-resistant endophytic bacterial mutants

The stability of the spontaneous antibiotic-resistant bacterial mutants were tested based on antibiotic resistance and *in vitro* antagonistic properties against *N. ditissima*.

To determine whether the antibiotic resistance was stably maintained, the spontaneous antibiotic-resistant mutants were subcultured onto NA in the absence of the selected antibiotic followed by three further subcultures. Then, the bacterial mutants were transferred onto NA containing the highest antibiotic concentration which the mutant strain was resistant to.

To determine where there was no change in the antagonistic properties of the antibiotic-resistant mutant strains, their ability to inhibit *N. ditissima* were compared to the relevant wild type using the dual-culture plating assay as described in the Section 3.2.4.

4.2.4 Genotyping of wild-type bacterial isolates and their spontaneous antibiotic mutant strains

In order to verify the mutants were derived from the selected wild-type bacterial isolates, the genotypes of the wild-type bacterial isolates and their spontaneous antibiotic mutant strain were confirmed using enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR). PCR was done using primers ERIC 1R (5'-ATGTAA GCT CCT GGG GAT TCA C-3') and ERIC 2 (5'-AAG TAA GTG A CT GGG GTGAGC G-3') as described by Versalovic et al. (1991). PCR were performed in a total reaction mixture of 20 µL consisting of 10 µL DreamTaq (Life Technologies, Thermo Fisher Scientific, USA), and 1 µL of 25 µM of ERIC 1R and 1 µL of 25 µM of ERIC 2. Bacterial DNA was extracted using Gentra PureGene (Qiagen, USA) following the manufacturer's instructions. One µL of each DNA sample at ~50 ng/µL was used as a template. PCR was performed in a thermal cycler using the following protocol: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min, with final extension step at 72°C for 10 min. A 5 µL aliquot of PCR product was separated by electrophoresis at 10 V/cm in a 1.0% agarose gel in 1 × TAE alongside the 1Kb plus DNA Ladder (Invitrogen, Thermo Fisher Scientific, USA). Gels were stained in ethidium bromide solution for 15 min, destained with water then visualised under ultra-violet light using the UVIreader (Uvitec, UK).

4.2.5 Growth rate measurement of the spontaneous antibiotic resistant endophytic bacterial mutants

To determine the bacterial cell concentration of the antibiotic-resistant mutants for inoculation purposes a growth rate regression line based on the optical density (OD) of the culture broth at 600 nm (OD600) and CFU/mL determined by serial dilution plating was generated.

A loopful of each bacterial mutant strain from the -80°C stocks was streaked across NA in the absence of antibiotics. The plates were incubated at 25°C in darkness for 3 days. A single colony for each isolate was inoculated into 1 mL NB in 1.7 mL tubes and incubated overnight (12-13 h) at 28°C on an orbital shaker at 180 rpm. Then, for each isolate 100 µL of the overnight culture was used to inoculated 10 mL NB in 50 mL tubes (NEST, Nest Biotechnology, China), with duplicate tubes for each isolate. The tubes were then incubated at 28°C on an orbital shaker at 180 rpm. A 1.1 mL sample from each bacterial culture was taken at 2 hourly intervals with 1 mL used to determine the OD at 600 nm measured using a spectrophotometer (Jenway, Model 6305, UK) and 100 µL used to determine the CFU/mL by serial dilution plating on NA. For serial dilution plating the culture broth was serially diluted using phosphate buffered saline (PBS, pH 7.2) and the dilutions spread plate on duplicate NA plates. After incubation at 25°C in darkness for 2-3 days the number of colonies were recorded and used to determine the CFU/mL. To enable the full growth curve to be determined, one set of duplicate tubes were set up to be sampled after 2 h, 4 h, 6 h, 8 h, 10 h and 12 h and the other set to be sampled after 12 h until the OD600 values became constant.

A standard growth curve for each bacterial mutant strain was produce by plotting the OD600 and CFU/mL data determined at each time point and the linear regression line produced in Excel (Wicaksono et al., 2018). For inoculum production for the subsequent experiment, the growth curve for each bacterial strain will be used to determine the appropriate time during the exponential growth stage to harvest the culture, with the equation for the regression line used to determine the inoculum concentration (CFU/mL) based on OD600 measurements.

4.2.6 Colonisation of detached 'Royal Gala' shoots by endophytic bacterial mutants in autumn 2017

The bacterial mutant strains with stable antibiotic resistance and antagonistic properties against *N. ditissima*, were selected for the experiment.

4.2.6.1 Plant material

The experiment was conducted using detached shoots of 'Royal Gala' collected in early April 2017, autumn in New Zealand. One-year old terminal shoots (approx. 25 cm long) with leaves and leaf buds and without any visible disease symptoms and obvious wounds were collected from 'Royal Gala' trees in the Lincoln University Research Orchard. The cut section of the collected shoots was placed in the water for 1 day before inoculation.

4.2.6.2 Inoculum preparation

A single colony of each bacterial mutant strain was inoculated into 1 mL NB and incubated at 28°C for ~12 h on an orbital shaker at 180 rpm. Then, 100 µL of the resulting bacterial culture was inoculated into 5 mL NB in 15 mL centrifuge tubes and incubated at 28°C on an orbital shaker at 180 rpm. Inoculum was produced by harvesting the bacteria at the exponential phase and by centrifugation at 4000 ×g for 10 min at 10°C in a refrigerated centrifuge (Wicaksono et al., 2017). The resulting pellet was resuspended in PBS (pH 7.2) to achieve a concentration of 10^6 CFU/mL based on the OD600 measurement.

4.2.6.3 Inoculation strategy

The middle internode of each shoot was surface sterilised by swabbing with a cotton swab dipped in 70% ethanol and a 3-mm wound was made with a sterile cork borer and a sterile scalpel. Ten µL of a 10^6 CFU/mL inoculum of one of the selected endophyte strain suspensions was used to inoculate the wound (10^4 CFU/wound). The wounds were then covered with Parafilm® (Bemis) 5 min after inoculation when the inocula had been absorbed into the wound. Shoots serving as negative controls were inoculated with 10 µL PBS (pH 7.2) instead of the bacterial inoculum. After inoculation, the base of the shoots were placed into 28 mL Universal bottles containing 20 mL water and liquid fertiliser (water: white Chrysal = 200: 1) and incubated on a bench under a light bank (Osram L36W.840 Lumilux Cool White lights (100-150 $\mu E.s^{-1}.m^{-2}$)) with 12 h dark/12 h light at 22°C (Figure 4.1). Eight replicates were set up for each treatment and assessment time, and the experiment was arranged in a randomised block design with one replicate per treatment in each block. The shoots were kept moist by spraying with tap water every day. The water/Chrysal solution was refreshed every week and a few millimetres of the shoot base were cut off to ensure the shoots were able to absorbed the solution effectively (Ghasemkhani et al., 2015).

Figure 4.1 Set up of the experiment to determine the colonisation of detached 'Royal Gala' shoots by endophytic bacterial mutants under a light bank.

4.2.6.4 Assessment

Plants were destructively harvested 14, 21 and 28 days after inoculation to determine endophyte colonisation of the detached shoot at the point of inoculation and 5 cm above and below the inoculation point. Eight replicate shoots were harvested after 14 and 21 days and 4 shoots after 28 days incubation to assess endophyte colonisation in 11 stem points (the inoculation point and every 1 cm, 2 cm, 3 cm, 4 cm and 5 cm above and below the inoculation point), with the remaining 4 shoots used to determine the population of the antibiotic resistant bacterial mutant strains colonising four stem points (1 and 3 cm above and below the inoculation point).

For each shoot, a 12 cm long stem segment comprising the inoculation point and 5 cm above and below the inoculation point was surface sterilised by soaking in 96% ethanol for 10 s, followed by 3 min in a 2.5% sodium hypochlorite solution and then rinsed 3 times in sterile water for 1 min each time in a laminar flow hood. From each shoot, 1 mm tissue pieces were aseptically dissected at each of the 11 assessment points which were 0 (inoculation point), 1 cm, 2 cm, 3 cm, 4 cm and 5 cm above and below the inoculation point. Then, the 1 mm tissue pieces were plated onto NA amended with 125 ppm Rifampicin and 50 ppm Cycloheximide (Sigma, Sigma-Aldrich, USA) (NA+Rif+Cyclo). The plates were incubated at 25°C in the darkness for 7 days, with the plates observed for presence of rifampicin-resistant bacterial colonies growing from the wood pieces after 7 days incubation.

The remaining four replicate shoots for the 28 days assessment were used to determine the population of the rifampicin resistant bacterial mutant strains colonising 1 cm stem sections at 1 cm and 3 cm above and below the inoculation point. The stems were surface sterilised as previously described and aseptically dissected into 1 cm section at each assessment point, with each 1 cm stem segment aseptically macerated using a sterile mortar and pestle in 10 mL sterile PBS (pH 7.2), then serially diluted to 10 and 100-fold using PBS (pH 7.2). One-hundred µL of the 10^0, 10^{-1} and 10^{-2} tissue extraction solution were spread plated on duplicate NA+Rif+Cyclo plates. The plates were incubated at 25°C in the darkness for 1 week after which the number of colonies were counted and used to determine the CFU/stem section.

In addition, a representative number of recovered bacterial colonies (n = 10-20) were randomly selected and subcultured onto NA. Bacterial DNA was extracted using Gentra PureGene (Qiagen, USA) following the manufacturer's instructions. Bacterial DNA was then genotyped using ERIC-PCR as described in Section 4.2.4, in order to verify they were the inoculated strain.

4.2.7 Colonisation of detached 'Royal Gala' shoots by endophytic bacterial mutants in spring-summer 2017

To determine whether season or apple shoot growth stage affected endophytic colonisation, the experiment was repeated with detached 'Royal Gala' shoots collected in November 2017 (spring-summer). The endophytic bacterial mutants which were shown to colonise the detached shoots in the experiment conducted in autumn 2017 (Section 4.2.6) were selected. The experiment was conducted as previously described in Sections 4.2.6.2 and 4.2.6.3, with the shoots harvested 14 and 26 days after inoculation. The assessment method was as described in the Section 4.2.6.4. Fourteen days after inoculation, all 8 replicate shoots per treatment were used to assess for endophyte colonisation at 11 assessment points. Twenty-six days after inoculation, four replicate shoots were assessed for endophyte colonisation in the 1 mm stem pieces cut from the 11 assessment points and the remaining four replicate shoots were used to determine endophyte population in the 1 cm stem sections at four assessment points. A representative number of recovered bacterial isolates (n = 5 from each bacterial mutant) were genotyped using ERIC-PCR as described in Section 4.2.4.

4.2.8 Data analysis

Number of stem pieces positive for bacterial growth in different antibiotic treated NA at three concentrations was analysed with one-way ANOVA followed by Fisher's protected LSD test at $p \leq 0.05$ in Minitab 17. Radial growth of three *N. ditissima* isolates was analysed with nonparametric Kruskal-Wallis one-way test at $p \leq 0.05$ in SPSS Statistics 24.

Movement and persistence of the inoculated bacterial endophyte was determined by the recovery of them from each assessment stem point at different assessment time points. Recovery frequency at each assessment stem point was determined by the percentage of the number of shoots they were recovered from the number of inoculated shoots.

4.3 Results

4.3.1 Production of spontaneous endophytic bacterial mutants

The mean number of apple stem pieces plated on NA amended with each concentration of each antibiotic that was positive for growth of background endophytic bacteria is presented in Table 4.1. No bacteria grew from the apple stem pieces plated on NA amended with 75 and 100 ppm rifampicin, with these being significantly different from the unamended NA control (Table 4.1; Appendix A4.1). Therefore, rifampicin was selected for producing mutants for the 16 selected antagonistic endophytic bacteria. Chloramphenicol was the second most effective antibiotic, showing no significant difference from rifampicin at any of the concentrations tested (Table 4.1; Appendix A4.1). Therefore, chloramphenicol was used to develop antibiotic resistant mutants for bacterial isolates for which attempts with rifampicin were unsuccessful.

Table 4.1 The mean number of apple stem pieces positive for background endophytic bacterial colonies growing on nutrient agar (NA) amended with four antibiotics at three concentrations (50 ppm, 75 ppm and 100 ppm).

	Mean number of stem pieces positive for bacterial growth[#]			
Antibiotic treatments	50 ppm	75 ppm	100 ppm	0 ppm
NA Control				1.7 abc
Streptomycin	1.7 abc	1.3 abcd	2.7 a	
Erythromycin	2.3 ab	0.7 cd	1.7 abc	
Chloramphenicol	0.7 cd	0.3 cd	1.0 bcd	
Rifampicin	0.7 cd	0.0 d	0.0 d	
p value	0.01*			

Mean values that do not share the same letter are significantly different according to one-way ANOVA followed by Fisher's protected LSD test at $p \leq 0.05$ as determined in Minitab 17. * significantly different ($p \leq 0.05$). [#] There were three replicate plates for each concentration of each antibiotic, with six stem pieces on each plate.

Of the 16 antagonistic endophytic bacterial isolates, six mutants resistant to 125 ppm rifampicin were produced, which included three *Bacillus* spp. isolates 42-1206(19)b$^{125ppmRif+}$, 21-606(28)b$^{125ppmRif+}$and R3L-6b$^{125ppmRif+}$, and three *Pseudomonas* spp. isolates 20-579(18)b$^{125ppmRif+}$, 7-208(18)b$^{125ppmRif+}$, and 31b3$^{125ppmRif+}$ (Table 4.2). For the 10 remaining endophytic bacteria strains,

five mutant strains with resistance up to 20 ppm or 75 ppm rifampicin were produced (4 *Pseudomonas* sp. isolates and 1 *Bacillus* sp. isolate). For the remaining five strains production of chloramphenicol mutant strains was attempted. For four of these isolates, mutant strains with resistance to 125 ppm chloramphenicol was successfully produced. They were *Bacillus* spp. isolates 26-771(23)b$^{125ppmChlo+}$, 41-1183(7)b$^{125ppmChlo+}$, 41-1183(6)b$^{125ppmChlo+}$ and 41-1182(4)b$^{125ppmChlo+}$. No mutant strains resistant to either 125 ppm rifampicin or 125 ppm chloramphenicol were produced for *Bacillus* sp. R1GS-12b (Table 4.2).

Table 4.2 Details of the most effective endophytic bacterial isolates at inhibiting the *in vitro* growth of three *Neonectria ditissima* isolates (ICMP14417, RS324p and MW15c1) used to produce spontaneous antibiotic resistant mutants (rifampicin-resistant (Rif+) and chloramphenicol-resistant (Chlo+) bacterial mutants) and the antibiotic concentration level (ppm) resistance achieved.

Relative ranking	Bacterial isolates	Percent inhibition (%)			Antibiotic resistance level
		ICMP14417	RS324p	MW15c1	
1	*Bacillus* sp. 42-1206(19)b	81.0	80.8	77.8	125 ppm Rif+
2	*Bacillus* sp. R1GS-12b	75.1	75.3	70.1	None
3	*Bacillus* sp. 26-771(23)b	77.0	80.2	59.2	125 ppm Chlo+
4	*Bacillus* sp. 21-606(28)b	68.6	67.8	55.0	125 ppm Rif+
5	*Bacillus* sp. 41-1183(7)b	50.1	40.4	68.4	125 ppm Chlo+
6	*Bacillus* sp. 41-1183(6)b	50.3	44.7	50.8	125 ppm Chlo+
7	*Bacillus* sp. 41-1182(4)b	49.3	33.2	65.7	125 ppm Chlo+
8	*Pseudomonas* sp. 20-579(18)b	39.8	37.3	34.6	125 ppm Rif+
9	*Pseudomonas* sp. 7-208(18)b	34.1	34.1	32.2	125 ppm Rif+
10	*Bacillus* sp. R3L-6b	42.4	29.6	26.8	125 ppm Rif+
11	*Pseudomonas* sp. 39-1143(30)b	33.6	30.7	21.4	20 ppm Rif+
12	*Pseudomonas* sp. 26-785(43)b	29.2	25.0	22.2	75 ppm Rif+
13	*Bacillus* sp. R3L-1b	44.0	13.4	18.7	20 ppm Rif+
14	*Pseudomonas* sp. 27-801(89)b	26.5	14.6	12.9	20 ppm Rif+
15	*Pseudomonas* sp. 31b3	21.3	14.4	7.2	125 ppm Rif+
16	*Pseudomonas* sp. 31b1	21.5	5.3	5.7	20 ppm Rif+

4.3.2 Stability tests of endophytic bacterial mutants

4.3.2.1 Stable maintenance of antibiotic resistance

The six mutant strains with resistance to 125 ppm rifampicin (42-1206(19)b$^{125ppmRif+}$, 21-606(28)b$^{125ppmRif+}$, R3L-6b$^{125ppmRif+}$, 20-579(18)b$^{125ppmRif+}$, 7-208(18)b$^{125ppmRif+}$ and 31b3$^{125ppmRif+}$) were shown to stably maintain resistance to 125 ppm rifampicin after successively being subcultured on NA without any antibiotic and then onto NA amended with 125 ppm rifampicin, having similar growth on both media (Figure 4.2). This indicated that resistance to 125 ppm rifampicin was stably maintained after several generations.

The four mutant strains with resistance to 125 ppm chloramphenicol (26-771(23)b$^{125ppmChlo+}$, 41-1183(7)b$^{125ppmChlo+}$, 41-1183(6)b$^{125ppmChlo+}$ and 41-1182(4)b$^{125ppmChlo+}$), however, were shown only to have weak growth on NA amended with 125 ppm chloramphenicol after successive subculturing on NA without any antibiotic indicating that resistance was not stably maintained.

Based on these results the six rifampicin-resistant mutants were selected for the remaining experiments.

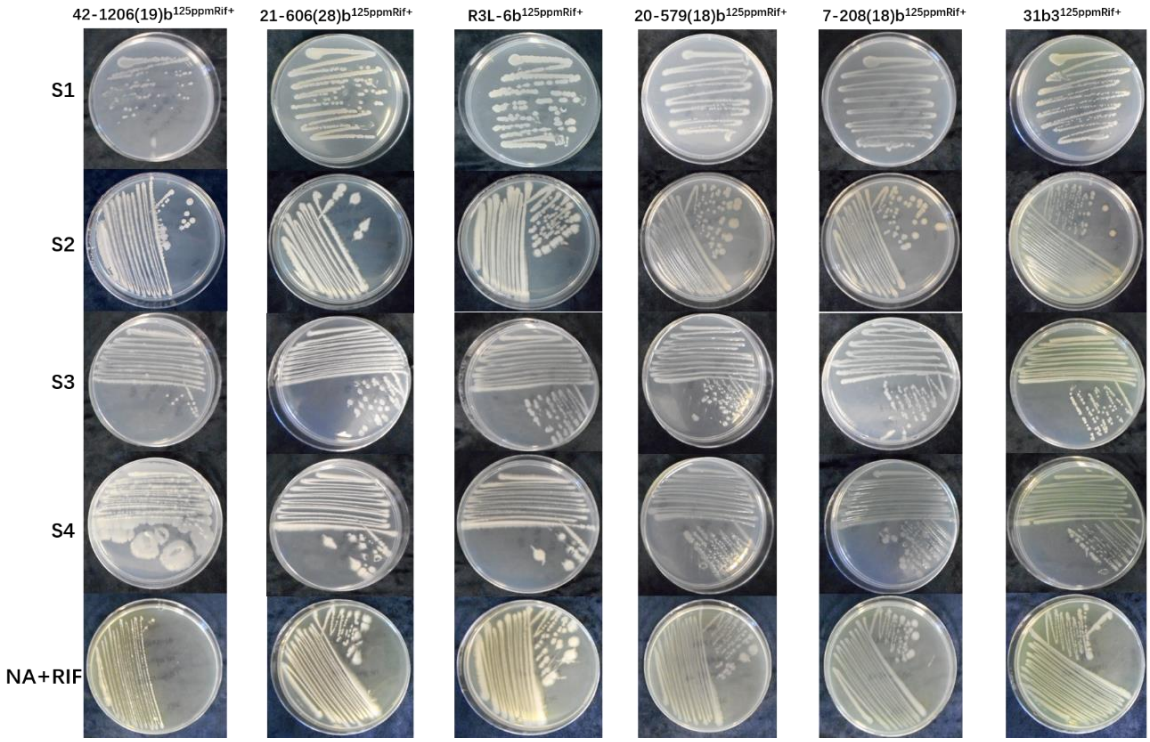

Figure 4.2 Comparison of the growth of six rifampicin-resistant mutant strains on nutrient agar (NA), and on NA amended with 125 ppm rifampicin (NA+RIF) after successive subculturing on NA.

4.3.2.2 *In vitro* biocontrol properties

There was no significant difference between the radial growth of each *N. ditissima* isolate inhibited by the wild type and the corresponding mutant of each of the six selected endophytic bacterial isolates. Wild type and mutant strains of *Bacillus* isolates 42-1206(19)b, 21-606(28)b and R3L-6b showed significant inhibition in radial growth of the three *N. ditissima* isolates as compared to the control ($p < 0.001$ for each *N. ditissima*, Table 4.3; Appendix A4.2).

Table 4.3 Radial growth (mm) of three *Neonectria ditissima* isolates (ICMP14417, RS324p and MW15c1) inhibited by wild type (WT) and mutant (MT) strains of six selected endophytic bacterial isolates in the dual culture plating assay after 17 days growth.

	Radial growth of *Neonectria ditissima* (mm)		
Bacterial isolate	ICMP14417	RS324p	MW15c1
*Ba.*42-1206(19)b (WT)	3.62 a	4.01 a	1.78 a
*Ba.*42-1206(19)b (MT)	3.44 a	4.30 ab	4.05 ab
*Ba.*21-606(28)b (WT)	6.11 ab	6.75 abc	3.71 abc
*Ba.*21-606(28)b (MT)	10.19 abcd	7.56 abc	8.81 abcd
*Ba.*R3L-6b (WT)	16.63 abcde	13.10 abcd	15.86 abcdef
*Ba.*R3L-6b (MT)	8.16 abc	9.39 abc	10.49 abcde
*Pse.*7-208(18)b (WT)	21.35 abcdef	19.49 cde	19.07 bcdefg
*Pse.*7-208(18)b (MT)	21.53 abcdef	19.76 cde	19.40 bcdefg
*Pse.*20-579(18)b (WT)	22.89 bcdef	19.48 bcde	19.83 cdefg
*Pse.*20-579(18)b (MT)	23.22 cdef	19.46 cde	20.30 defg
*Pse.*31b3 (WT)	26.47 def	23.04 de	26.58 efg
*Pse.*31b3 (MT)	27.79 ef	23.42 de	27.58 fg
Control	33.03 f	29.34 e	30.22 g
p value	< 0.001**	< 0.001**	< 0.001**

Means followed by the same letter within a column are not significantly different based on pairwise comparisons using nonparametric Kruskal-Wallis one-way test, respectively. ** Highly significantly different ($p \leq 0.005$). *Ba.* and *Pse.* refer to *Bacillus* sp. and *Pseudomonas* sp., respectively.

4.3.3 Genotyping of wild-type bacterial isolates and their spontaneous produced mutant strains

As shown in Figure 4.3, the mutant strains showed the same ERIC-PCR genotype patterns as the corresponding wild-type isolates. Based on these results, the ERIC-PCR will be used to confirm the identity of the bacteria recovered from the inoculated shoots as the inoculated strains.

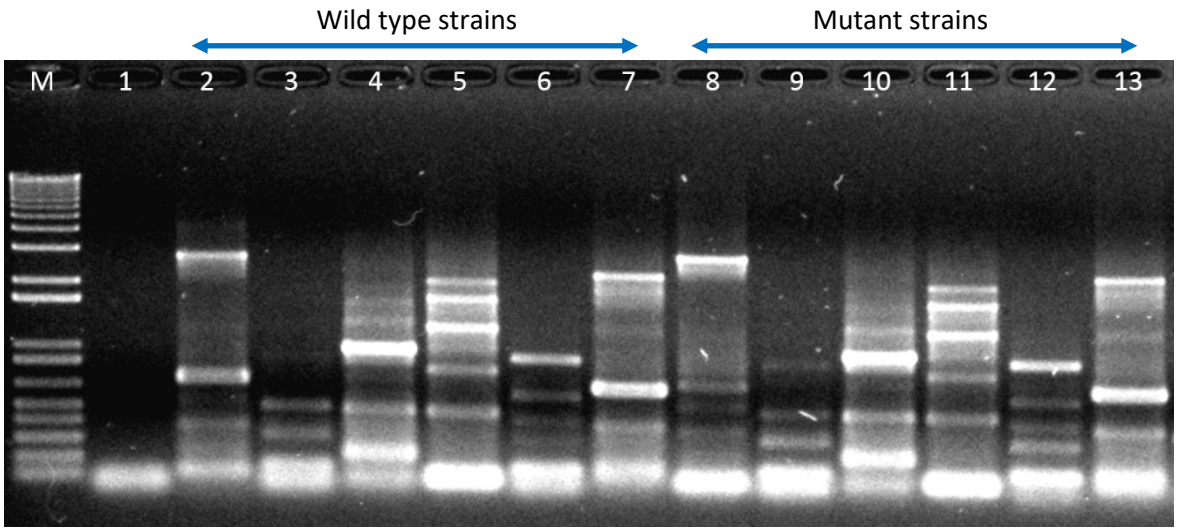

Figure 4.3 Agarose gel showing genotypes of the six rifampicin-resistant mutants (MT) and their respective wild types (WT) by ERIC PCR. M: 1 kb plus ladder (Invitrogen), 1: negative control (sterile PCR water), 2-7: WT42-1206(19)b, WT21-606(28)b, WT20-579(18)b, WT7-208(18)b, WTR3L-6b and WT31b3, 8-13: MT42-1206(19)b, MT21-606(28)b, MT20-579(18)b, MT7-208(18)b, MTR3L-6b and MT31b3.

4.3.4 Colonisation of detached 'Royal Gala' shoots by endophytic bacterial mutants in 2017

A standard growth curve (OD600 and CFU/mL) was generated for each of the six rifampicin-resistant mutant strains (Appendix A4.3-A4.8).

4.3.4.1 Colonisation of endophytic bacterial mutants in detached shoots of 'Royal Gala' in autumn 2017

Apart from a minor wound response around the inoculation point no lesions developed around the point of inoculation and the leaves and stems appeared healthy with no apparent symptoms, indicating these endophytes were non-pathogenic. Colonisation of stem tissue was assessed at 14, 21 and 28 days after inoculation. No bacterial colonies were recovered on the rifampicin resistant amended agar from any of the control shoots. Bacterial colonies were recovered on the antibiotic amended agar from the shoots inoculated with three *Pseudomonas* spp. isolates (20-579(18)b$^{125ppmRif+}$, 7-208(18)b$^{125ppmRif+}$ and 31b3$^{125ppmRif+}$) and one *Bacillus* sp. isolate (42-

1206(19)b$^{125ppmRif+}$) after 14, 21 and 28 days. Rifampicin resistant bacterial colonies were recovered from some but not all replicates of the shoots inoculated with the four bacterial mutant treatments for each of the assessed stem distances from the inoculation point and were generally recovered from the stem tissue 4-5 cm above and below the inoculation point 14 days after inoculation. Rifampicin resistant bacterial colonies were successfully recovered from the stem tissue 4-5 cm above and below the inoculation point 28 days after inoculation (Table 4.4). For the apple shoots inoculated with the other two *Bacillus* spp. isolates, 21-606(28)b$^{125ppmRif+}$ and R3L-6b$^{125ppmRif+}$, no resistant bacterial colonies were recovered from any of the 11 assessed stem points at the three assessment time.

At 28 days after inoculation, four replicate shoots for the four bacterial mutants (42-1206(19)b$^{125ppmRif+}$, 20-579(18)b$^{125ppmRif+}$, 7-208(18)b$^{125ppmRif+}$ and 31b3$^{125ppmRif+}$) were assessed to determine the population level colonising the detached shoots at 1 cm and 3 cm above and below the inoculation point. Rifampicin resistant bacterial colonies were only recovered from one replicate shoot inoculated with isolate 20-579(18)b$^{125ppmRif+}$, with CFU counts between 11,500 and 166,00 CFU/cm in stem segment recovered from 3 cm, 1 cm above and 1 cm below the inoculation point (Table 4.5). No colonies were recovered on the plates for the other replicates, and the other bacterial isolates, indicating that the number of colonies in the stem segment was lower than the threshold value of 3,000 CFU/cm (1 cm stem segment crushed in 10 mL and 100 µL plated) for detection by this method.

A representative number of the recovered bacterial colonies (n = 14-20) from different shoot replicates and different time assessments were identified by genotyping using ERIC PCR with the fingerprints compared with the pure bacterial cultures used for inoculation. Colonies recovered from the shoots inoculated with 20-579(18)b$^{125ppmRif+}$, 7-208(18)b$^{125ppmRif+}$ and 31b3$^{125ppmRif+}$ were confirmed to be the inoculated isolates as they showed the same fingerprints as the pure strains used for inoculation (Figure 4.4b, c, d). However, for bacteria recovered from shoots inoculated with isolate 42-1206(19)b$^{125ppmRif+}$ the fingerprint did not correspond to that of the pure strain used for inoculation (Figure 4.4a), and was similar to the banding pattern for isolate 20-579(18)b$^{125ppmRif+}$ (Figure 4.4d).

Table 4.4 Percentage frequency of rifampicin-resistant bacterial mutant colonies recovered from the inoculation point and from the 1 cm, 2 cm, 3 cm, 4 cm, 5 cm above (+) and below (-) the inoculation point in eight replicate apple shoots assessed 14 days and 21 days after inoculation and four replicate shoots assessed 28 days after inoculation with six endophytic antibiotic resistant mutant bacterial isolates. Shaded squares indicate stem segments where rifampicin-resistant bacterial mutants were isolated.

Isolate	Days (d)	Percentage recovery of resistant bacterial colonies from stem tissue segments (April-May 2017)										
		+5 cm	+4 cm	+3 cm	+2 cm	+1 cm	0 cm	-1 cm	-2 cm	-3 cm	-4 cm	-5 cm
Pseudomonas sp. 20-579(18)b$^{125ppmRif+}$	14 d	0%	25%	38%	63%	63%	88%	38%	63%	38%	50%	38%
	21 d	0%	0%	13%	13%	50%	88%	38%	38%	25%	0%	0%
	28 d	50%	50%	25%	75%	75%	75%	50%	25%	25%	25%	75%
Pseudomonas sp. 7-208(18)b$^{125ppmRif+}$	14 d	63%	63%	63%	75%	100%	88%	75%	88%	75%	75%	75%
	21 d	13%	0%	38%	25%	50%	88%	38%	13%	25%	13%	13%
	28 d	0%	25%	25%	75%	100%	75%	75%	50%	75%	50%	50%
Pseudomonas sp. 31b3$^{125ppmRif+}$	14 d	38%	25%	25%	63%	88%	88%	63%	63%	38%	38%	38%
	21 d	38%	13%	38%	63%	75%	100%	75%	25%	38%	25%	38%
	28 d	25%	0%	25%	75%	75%	100%	75%	75%	25%	25%	25%
Bacillus sp. 42-1206(19)b$^{125ppmRif+}$	14 d	0%	25%	38%	63%	75%	88%	50%	75%	50%	50%	50%
	21 d	0%	13%	13%	50%	25%	75%	38%	50%	38%	25%	25%
	28 d	0%	25%	0%	50%	25%	100%	50%	75%	50%	25%	50%
Bacillus sp. 21-606(28)b$^{125ppmRif+}$	14 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	21 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	28 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Bacillus sp. R3L-6b$^{125ppmRif+}$	14 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	21 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	28 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Table 4.5 Number of bacterial colonies (CFU/cm of stem) recovered on rifampicin amended agar from the stem segments 1 and 3 cm above (+) and below (-) the inoculation point for the four replicates shoots for each of the four rifampicin-resistant bacterial mutant strains used for inoculation, 28 days after inoculation.

Isolate	Replicate shoot	Number of bacterial colonies recovered (CFU/cm)			
		+3 cm	+1 cm	-1 cm	-3 cm
20-579(18)b$^{125ppmRif+}$	1	11,500	145,000	166,000	0
20-579(18)b$^{125ppmRif+}$	2	0	0	0	0
20-579(18)b$^{125ppmRif+}$	3	0	0	0	0
20-579(18)b$^{125ppmRif+}$	4	0	0	0	0
7-208(18)b$^{125ppmRif+}$	1	0	0	0	0
7-208(18)b$^{125ppmRif+}$	2	0	0	0	0
7-208(18)b$^{125ppmRif+}$	3	0	0	0	0
7-208(18)b$^{125ppmRif+}$	4	0	0	0	0
31b3$^{125ppmRif+}$	1	0	0	0	0
31b3$^{125ppmRif+}$	2	0	0	0	0
31b3$^{125ppmRif+}$	3	0	0	0	0
31b3$^{125ppmRif+}$	4	0	0	0	0
42-1206(19)b$^{125ppmRif+}$	1	0	0	0	0
42-1206(19)b$^{125ppmRif+}$	2	0	0	0	0
42-1206(19)b$^{125ppmRif+}$	3	0	0	0	0
42-1206(19)b$^{125ppmRif+}$	4	0	0	0	0

‘0’ indicates that the number of bacterial colonies is lower than the detection threshold value (3,000 CFU/cm).

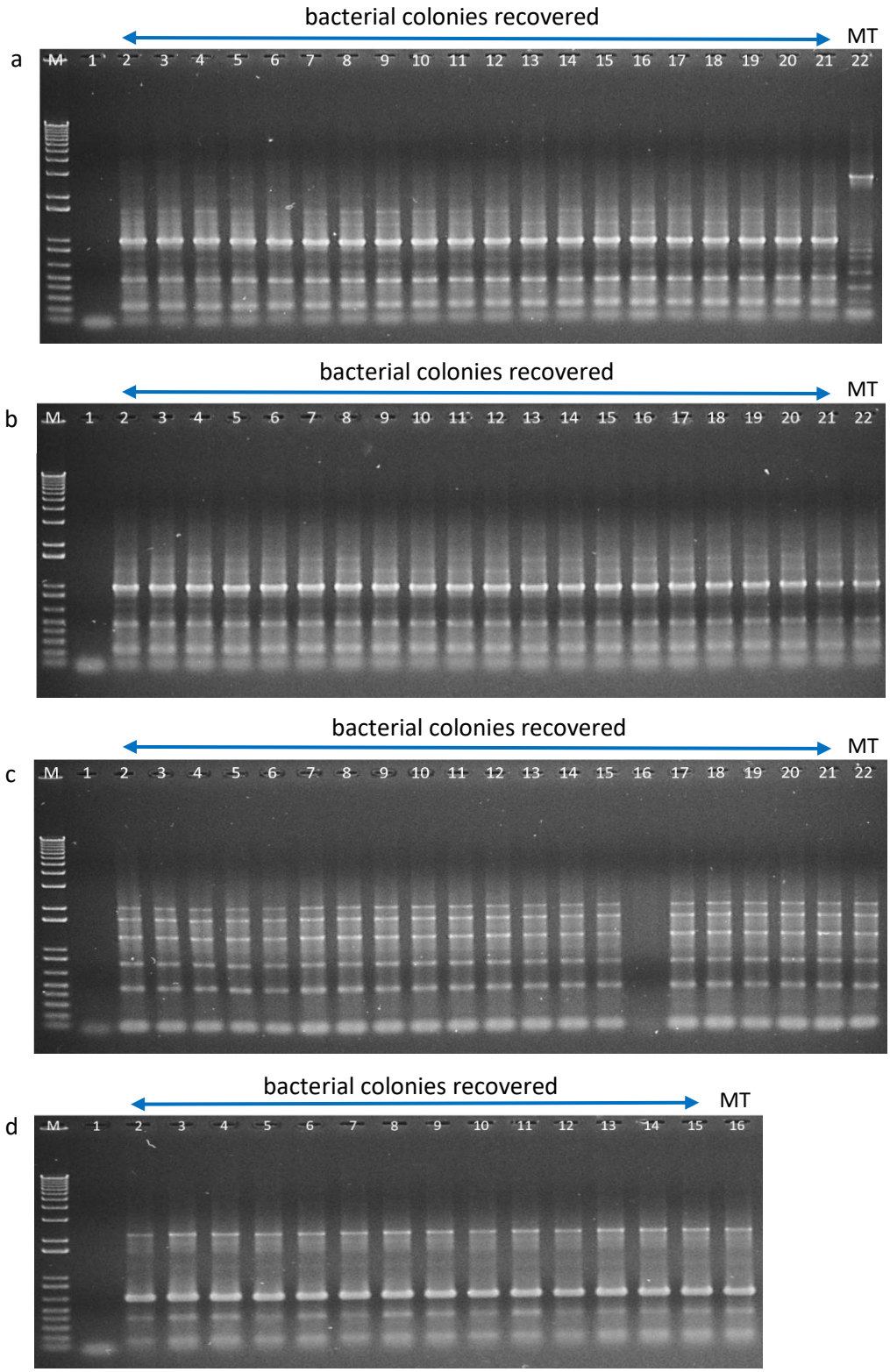

Figure 4.4 Agarose gel showing genotypes of the bacterial colonies recovered from the apple shoots inoculated with the four rifampicin-resistant mutants compared with that of a pure culture of the mutants (MT). M: 1 kb plus ladder (Invitrogen), 1: negative control (sterile PCR water). a) 2-21: bacterial colonies recovered from the shoots inoculated with 42-1206(19)$^{125ppmRif+}$, 22: 42-1206(19)$^{125ppmRif+}$; b) 2-21: bacterial colonies recovered from the shoots inoculated with 20-579(18)$^{125ppmRif+}$, 22: 20-579(18)$^{125ppmRif+}$; c) 2-21: bacterial colonies recovered from the shoots inoculated with 7-208(18)$^{125ppmRif+}$, 22: 7-208(18)$^{125ppmRif+}$; d) 2-15: bacterial colonies recovered from the shoots inoculated with 31b3$^{125ppmRif+}$, 16: 31b3$^{125ppmRif+}$.

4.3.4.2 Colonisation of endophytic bacterial mutants in detached shoots of 'Royal Gala' in spring-summer 2017

The ability of the three endophyte mutants strains, 20-579(18)b$^{125ppmRif+}$, 7-208(18)b$^{125ppmRif+}$ and 31b3$^{125ppmRif+}$, which were shown to colonise the apple shoots in the previous experiment conducted in autumn 2017 were determined in a repeat experiment carried out in spring-summer 2017. As the result of isolate 42-1206(19)b$^{125ppmRif+}$ was compromised in the previous experiment, due to the inoculum being contaminated, this isolate was included again in this experiment. Rifampicin resistant bacterial colonies were recovered from shoots inoculated with 20-579(18)b$^{125ppmRif+}$, 7-208(18)b$^{125ppmRif+}$ and 31b3$^{125ppmRif+}$ 4-5 cm above and below the inoculation wound 14 and 26 days after inoculation. Antibiotic resistance colonies were recovered from some but not all replicates of the stems inoculated with the three bacterial mutant treatments for each of the assessed stem distances from the inoculation point. Recovery frequency generally decreased as the distance from the inoculation point increased (Table 4.6). Representative number of bacterial colonies (n = 5) from different shoot replicates and different time assessments were detected by ERIC PCR and were confirmed to be the inoculated isolates as they showed the same fingerprints as the pure strains used for inoculation, except that one bacterial colony recovered from shoot inoculated with 20-579(18)$^{125ppmRif+}$ showed to be contaminated by 7-208(18)$^{125ppmRif+}$. Banding pattern of the bacterial colonies is the same across all the replicates (Figure 4.5).

Rifampicin resistant bacterial colonies also grew from shoots inoculated with 42-1206(19)b$^{125ppmRif+}$ at the inoculation point (from 25% shoots), 1 cm and 3 cm below the inoculation point (from 25% and 13% shoots, respectively) 14 days after inoculation, and from negative control shoots at the inoculation point (from 13% shoots) and 1 cm above the inoculation point (from 13% shoots) 26 days after inoculation (Table 4.6). The ERIC-PCR result showed that the one bacterial colony from shoot inoculated with 42-1206(19)b$^{125ppmRif+}$ did not correspond with the pure strain and had a same band pattern as the two bacterial colonies recovered from the negative control shoot (Figure 4.5: 20-23). Therefore, they were identified as being a background bacterial endophyte from the apple shoots.

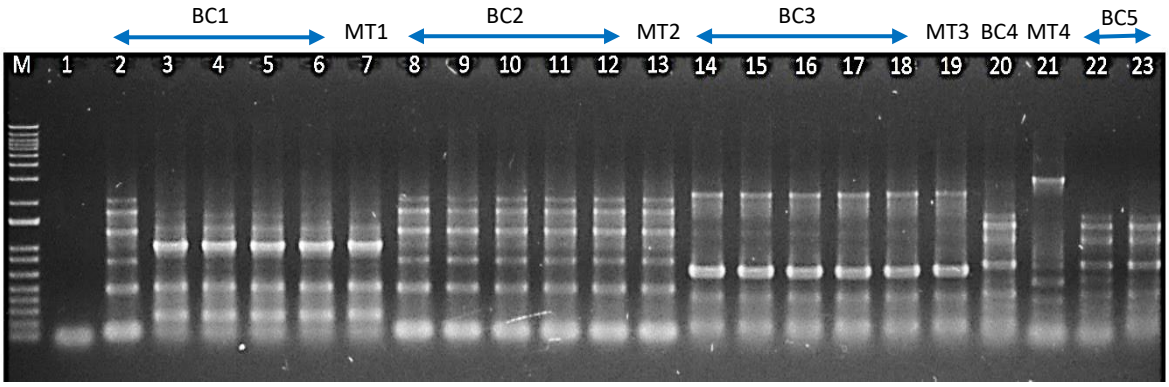

Figure 4.5 Agarose gel showing genotypes of the bacterial colonies recovered from the apple shoots inoculated with the four rifampicin-resistant mutants compared with that of a pure culture of the mutants. M: 1 kb plus ladder (Invitrogen), 1: negative control (sterile PCR water), 2-6 (BC1): bacterial colonies recovered from the shoots inoculated with 20-579(18)$^{125ppmRif+}$, 7 (MT1): 20-579(18)$^{125ppmRif+}$, 8-12 (BC2): bacterial colonies recovered from the shoots inoculated with 7-208(18)$^{125ppmRif+}$, 13 (MT2): 7-208(18)$^{125ppmRif+}$, 14-18 (BC3): bacterial colonies recovered from the shoots inoculated with 31b3$^{125ppmRif+}$, 19 (MT3): 31b3$^{125ppmRif+}$, 20 (BC4): a bacterial colony recovered from the shoots inoculated with 42-1206(19)$^{125ppmRif+}$, 21 (MT4): 42-1206(19)$^{125ppmRif+}$, 22-23 (BC5): bacterial colonies recovered from the negative control shoots.

Table 4.6 Percentage frequency of rifampicin resistant bacterial colonies recovered from stem segments above (+) and below (-) the inoculation wound (0 cm) in eight replicate apple shoots assessed 14 days after inoculation and four replicate shoots assessed 26 days after inoculation with four endophytic antibiotic resistant mutant bacterial isolates. Shaded squares indicate stem sections where rifampicin-resistant bacterial colonies were isolated.

Isolate	Days (d)	Percentage recovery of resistant bacterial colonies from stem tissue segments (Nov.-Dec. 2017)										
		+5 cm	+4 cm	+3 cm	+2 cm	+1 cm	0 cm	-1 cm	-2 cm	-3 cm	-4 cm	-5 cm
*Negative control	14 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	26 d	0%	0%	0%	0%	13%	13%	0%	0%	0%	0%	0%
Pseudomonas sp. 20-579(18)b$^{125ppmRif+}$	14 d	25%	13%	38%	38%	75%	75%	63%	63%	75%	38%	13%
	26 d	0%	25%	0%	0%	75%	100%	100%	75%	50%	0%	0%
Pseudomonas sp. 7-208(18)b$^{125ppmRif+}$	14 d	13%	38%	63%	63%	88%	100%	88%	75%	50%	63%	25%
	26 d	100%	100%	100%	75%	100%	100%	100%	75%	100%	100%	100%
Pseudomonas sp. 31b3$^{125ppmRif+}$	14 d	0%	13%	25%	63%	88%	100%	88%	63%	25%	13%	25%
	26 d	50%	75%	50%	75%	75%	100%	75%	50%	50%	50%	50%
# *Bacillus* sp. 42-1206(19)b$^{125ppmRif+}$	14 d	0%	0%	0%	0%	0%	25%	25%	0%	13%	0%	0%
	26 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

* ERIC PCR indicated that they did not correspond with any of the inoculating isolates. # ERIC PCR showed that they were not the inoculating isolate.

At 26 days after inoculation, the population level (CFU) of the three bacterial mutant strains, 20-579(18)$^{125ppmRif+}$, 7-208(18)$^{125ppmRif+}$ and 31b3$^{125ppmRif+}$, which were shown to colonise the detached shoots 14 days after inoculation were assessed. Rifampicin resistant bacterial colonies were recovered from some but not all replicates of the stems inoculated with the three bacterial mutant treatments for each of the assessed stem distances from the inoculation point. The population level of 7-208(18)$^{125ppmRif+}$ decreased as the distance from the inoculation point increased. However, this was not seen for 20-579(18)$^{125ppmRif+}$ and 31b3$^{125ppmRif+}$ (Table 4.7). No colonies were recovered on the plates for the other replicates, indicating that the number of colonies in the stem segment was lower than the threshold value of 1,500 CFU/cm (1 cm stem piece crushed in 5 mL and 100 µL plated) for detection by this method. The results indicated that strains 20-579(18)$^{125ppmRif+}$ and 7-208(18)$^{125ppmRif+}$ were more effective at colonising the detached shoots compared with strain 31b3$^{125ppmRif+}$ (Table 4.7).

Table 4.7 Number of colonies of the three tested rifampicin-resistant bacterial mutants recovered from four stem segments above and below inoculation wound on the shoots (four replicates) on the 26 days after inoculation.

Isolate	Replicate shoot	Number of bacterial colonies recovered (CFU/cm)			
		+3 cm	+1 cm	-1 cm	-3 cm
20-579(18)b$^{125ppmRif+}$	1	0	2,300	0	0
20-579(18)b$^{125ppmRif+}$	2	0	0	3,250	19,000
20-579(18)b$^{125ppmRif+}$	3	0	0	0	0
20-579(18)b$^{125ppmRif+}$	4	0	0	0	0
7-208(18)b$^{125ppmRif+}$	1	0	7,500	0	0
7-208(18)b$^{125ppmRif+}$	2	0	0	0	0
7-208(18)b$^{125ppmRif+}$	3	1,650	8,550	7,800	0
7-208(18)b$^{125ppmRif+}$	4	0	0	17,000	4,800
31b3$^{125ppmRif+}$	1	0	0	0	0
31b3$^{125ppmRif+}$	2	0	0	0	0
31b3$^{125ppmRif+}$	3	0	0	0	0
31b3$^{125ppmRif+}$	4	0	0	19,000	0

'0' indicated that the number of bacterial colonies is lower than the detection threshold value (1,500 CFU/cm).

4.4 Discussion

In this chapter, spontaneous bacterial mutants with resistance to 125 ppm rifampicin (Rif^{125ppm} strains) were successfully produced and used as inocula for colonisation assays in detached apple shoots. It enabled the inoculated endophytes to be specifically reisolated and the ability of the endophytes to colonise apple stem tissues to be determined. With the use of spontaneous bacterial mutants followed by ERIC-PCR genotyping confirmation, three Rif^{125ppm} strains derived from *Pseudomonas* isolates selected in this study showed colonisation and movement inside the detached apple shoots. This is the first study to evaluate the colonisation of potential biocontrol agents for European canker in apple in New Zealand.

Rifampicin mutants of all *Pseudomonas* isolates were able to be produced, although some had lower resistance. However, some of the *Bacillus* isolates failed to produce mutants with rifampicin resistance, while production of chloramphenicol resistance was successful. Resistance to rifampicin is conferred by mutation in the *rpoB* gene coding for mRNA polymerase or by alternations in membrane permeability (Chandrasekaran & Lalithakumari, 1998; Goldstein, 2014; Lambert, 2005). Point mutation of the *rpoB* gene is a common mechanism of spontaneous rifampicin mutant development and was found in these two genera (Hosokawa et al., 2002; Vogler et al., 2002). Failure in developing rifampicin resistance in some *Bacillus* isolates is likely because no point mutation occurred at the target site of rifampicin. Another possible reason for the unsuccessful production of rifampicin mutants for some *Bacillus* isolates, as well as for *Pseudomonas* and *Bacillus* isolates with lower resistance, is the selection system in this experiment did not allow the Rif^{125ppm} mutants to be produced. A narrower increase of rifampicin concentrations could be tried such as starting from 5 ppm and then increasing by 5 ppm in each of the next selection concentration.

All *Pseudomonas* spp. developed rifampicin resistance in this study. This could be explained by the known mechanism of membrane modification found in species of Gram-negative bacteria (Chandrasekaran & Lalithakumari, 1998; Ferenci & Phan, 2015; Pages et al., 2008). *Pseudomonas* species have intrinsic resistance to hydrophilic solutes such as those that deliver antibiotics, as their outer membrane forms a permeability barrier (Burns & Clark, 1992; Delcour, 2009). Normally, hydrophilic solutes, including those that deliver nutrients, cross the outer membranes through pores (small and large) produced by proteins called porins. However, under stressful conditions, such as the high rifampicin concentrations used for mutant production, there is selective pressure to reduce expression of outer-membrane porin proteins, especially large porins (Ferenci & Phan, 2015). As has been shown for other antibiotics, this may restrict rifampicin entry

producing resistance to a higher rifampicin concentration (125 ppm). Similar to the findings from Lavigne et al. (2013) that the selective pressure from carbapenems reduced porin expression in *Enterobacter aerogenes* and resulted in emergence of the carbapenems resistance isolate in *E. aerogenes*.

The use of spontaneous antibiotic resistant mutants facilitated monitoring the colonisation of the bacteria, because they can be specifically reisolation from inoculated plants onto antibiotic amended agar. The benefit of this technology is that only the living bacterial mutants are isolated, showing the survival and colonisation of the inoculated bacterial mutants in plants. Also, it is a more cost efficient method for producing mutants as a marker for monitoring the bacterial colonisation than other molecular technologies, such as tagging of a bacterial inoculum with non-antibiotic resistance marker genes which also allows survival and activity of the inoculum to be monitored (Björklöf & Jørgensen, 2001). However, there are some limitations in this technology. First, the spontaneous mutation was uncontrolled. The derived strains could have different mutation points from a same parent strain. For example, three mutations were reported in the *rpoB* gene and it resulted in different extent of fitness loss among them as compared to the wild type (Mariam et al., 2004). Second, the spontaneous mutation has the risk of reducing the fitness and altering metabolism such as porin production of the bacteria as it has been reported by previous studies (Lavigne et al., 2013; Linkevicius et al., 2013). Spontaneous point mutations can also influence the production of biologically active secondary metabolites. For example, streptomycin resistance in *Pectobacterium carotovorum* conferred by mutation in the *rpsL* gene caused the loss of carbapenem production (Barnard et al., 2010). For this reason, *in vitro* antagonistic activity of the bacterial mutants against *N. ditissima* was tested in the dual culture plate assay compared with the wild-type strains in this study. Further, mutants may lose the antibiotic resistance after being inoculated into plants or soil, which will cause underestimation of the colonisation ability of the bacterial strain when they are isolated onto the antibiotic amended agar. For example, reduced resistance to rifampicin was found by Compeau et al. (1988) for *Peudomonas fluorescens* and *P. putida* in inoculated soil. To avoid these limitations, other methods for tracking and monitoring inoculum can be used such as green fluorescent protein (GFP), enzyme-linked immunosorbent assay (ELISA) and quantitative PCR (qPCR) (Rilling et al., 2019; Ryan et al., 2008).

Maintenance of resistance to 125 ppm rifampicin/chloramphenicol was observed for Rif^{125ppm} strains (3 *Pseudomonas* and 3 *Bacillus* strains), but not for $Chlo^{125ppm}$ strains (4 *Bacillus* strains). In contrast, spontaneous mutant strains with resistance to 100 ppm chloramphenicol generated from *Pseudomonas* spp. maintained resistance after being subcultured five times on the agar in

the absence of chloramphenicol (Wicaksono et al., 2018). This difference is probably because the stability and maintenance of antibiotic resistance varies between bacterial species, which was also found by Griffiths et al. (1990) in *Escherichia coli*. The reduction in antibiotic resistance level associated with subculturing in the current study is probably due to the absence of selection pressure when subcultured on NA without chloramphenicol for several generations. Park et al. (2003) reported the absence of sustained antibiotic pressure in aquatic environments may result in coliform bacteria losing antibiotic-resistant gene cassettes of class 1 integrons. The reduction in resistance to 125 ppm chloramphenicol was likely to be permanent as the mutants grew well on NA without chloramphenicol, as it is outlined by Griffiths et al. (1990) that bacteria which fail to grow on antibiotic amended media after being resuscitated on media without the antibiotic indicated a permanent loss of resistance. The stability of antibiotic resistance was important as it verified that the lower recovery of the inoculant was not as a result of the bacterial mutant strains not being able to grow on the antibiotic amended agar due to loss or reduction in resistance. For this reason, only the six Rif^{125ppm} strains were selected for assessing their ability to colonise detached apple shoots. To enable the colonisation ability of the remaining isolates to be evaluated further work to determine whether the four Chlo^{125ppm} strains maintain stable resistance to lower concentrations of chloramphenicol (eg. 100 ppm and 75 ppm) which could be then used to selectively recover the mutant strains from apple shoots.

Rif^{125ppm} strains were similar to their respective wild types in their ability to inhibit the *in vitro* growth of *N. ditissima* isolates and had the same genotype detected by ERIC-PCR. As spontaneous mutants may not be identical to the wild type parent, as discussed previously, mutants are normally compared with their wild type strains for desired traits such as *in vitro* and *in vivo* antagonism to the target fungal pathogens and colonisation ability in plants (Bacon & Hinton, 2002; Lin et al., 2009; Wicaksono et al., 2017). Similar to this study, maintenance of *in vitro* antagonism was also found for rifampicin mutants spontaneously produced from *Bacillus subtilis* against *Verticillium dahliae* causing eggplant (*Solanum melongena*) verticillium wilt (Lin et al., 2009) and *Pseudomonas* strains against *Neofusicoccum luteum* and *N. parvum* in grapevine (*Vitis vinifera*) (Wicaksono et al., 2017). The consistent genotype and the *in vitro* biocontrol activity between wild type and mutant strains allowed the mutant strains to be used as inocula for determining colonisation ability in apple shoots. The spontaneous mutations did not change the genotype of the six bacterial isolates. This also confirmed that the single mutant bacterial colony selected represented its genotype and there had been no contamination during the culturing process.

In this study, only the *Pseudomonas* sp. (n = 3) but not the *Bacillus* sp. (n = 3) Rif^{125ppm} strains were found to colonise the detached shoots. A similar result was reported by Germaine et al. (2004) who isolated three endophytic *Pseudomonas* strains from xylem sap of poplar trees (*Populus*), with two of them able to colonise both roots and stems when they were inoculated to poplar cuttings. It showed the different species/strains had different ability to colonise plant tissues even when they were inoculated into the original host. Therefore, one of the possible reasons for colonisation inability by *Bacillus* isolates in this study could be they are plant tissue specific. The three *Pseudomonas* isolates in this study originated from stems of 'Braeburn', 'Scifresh' and 'Grimes Golden' respectively, while the three *Bacillus* isolates were from the leaves of 'Royal Gala' and 'Braeburn' which is a different tissue origin when they were inoculated on apple stem (Chapter 3). In contrast, West et al. (2010) reported a rifampicin-resistant *Bacillus cereus* mutant isolated from vine samples could colonise leaves of grapevine and further down the vine shoots when it was inoculated onto the leaf by placing inoculum suspension on the leaf surface. Both *Bacillus* and *Pseudomonas* species are reported to be competent endophytes, but this is plant host dependent (Hardoim et al., 2008; Rosenblueth & Martínez-Romero, 2006). The strong endophytic colonisation capacity of *Pseudomonas* Rif^{125ppm} strains was revealed by their ability to colonise shoots collected from both spring and autumn, irrespective of shoot age which may have affected the indigenous endophyte communities as it was found in Chapter 2 of this study and by Guo et al. (2008). Further, failure to reisolate the inoculated *Bacillus* Rif^{125ppm} strains could be because their resistance to rifampicin reduced so they no longer grew on agar amended with rifampicin at 125 ppm. Reduced rifampicin resistance was found for *Peudomonas fluorescens* and *P. putida* mutant strains with different levels when they were recovered from the inoculated soil, although they both were selected on NA amended with 100 ppm rifampicin (Compeau et al., 1988). It means stably maintained resistance on agar may not reflect the maintenance of resistance when colonising apple shoots. Also, the stable maintenance of resistance could vary between different bacterial genera, species and strains. The loss of antibiotic resistance could be due to the need to compete with indigenous endophytes, or because of the lack of selective pressure to retain the antibiotic resistance. Antibiotic resistance can be a metabolic burden for a microbe and result in decreased competitive fitness compared to the wild type strain (Compeau et al., 1988) or other antibiotic-susceptible bacterial strains (Yin et al., 2019). Therefore, future work could focus on developing qPCR to track these microbes in apple shoots to determine whether they are still present but may have lost antibiotic resistance.

The three *Pseudomonas* Rif^{125ppm} strains (20-579(18)$^{125ppmRif+}$, 7-208(18)$^{125ppmRif+}$ and 31b3$^{125ppmRif+}$) persisted in detached apple stems for up to 1 month after inoculation and also moved from the

inoculation point. Introduced endophytic bacteria were reported to persist in various plant tissues such as roots, leaves and stems, with the colonisation ability determined by various tracking methods such as antibiotic-resistant mutants (Lang et al., 2018; Wicaksono et al., 2018; Wicaksono et al., 2017) and confocal laser scanning microscopy (Mercado-Blanco et al., 2016). In this study, the three mutant strains colonised the inoculation wound and moved internally to colonise the tissue 4-5 cm above and below the inoculation wound. A similar result was also found for *Pseudomonas* Rif^{100ppm} strains in grapevine trunks (Wicaksono et al., 2017) and for *Pseudomonas* Chlo^{100ppm} strains in kiwifruit stems (Wicaksono et al., 2018). Moreover, recovery frequency generally decreased as the distance from the wounding inoculation point increased in the two colonisation experiments. The strong ability of the strains to colonise the tissue at the wound site is a critical factor in enabling the stains to provide wound protection against *N. ditissima*. Whether the three *Pseudomonas* Rif^{125ppm} strains persisted for longer than 1 month after inoculation and their potential spread in stem tissue further than 5 cm above/below the inoculation point, and their potential to move into fruit, to provide protection against the pathogen is unknown. Systemic colonisation of tomato roots, stem and leaves by *B. amyloliquefaciens* subsp. *plantarum* strain 32a was found from 15 to 60 days after seed inoculation (Abdallah et al., 2019). However, whether further study on the persistence and movement of the three *Pseudomonas* Rif^{125ppm} strains within the apple tissue is required will depend on whether any of the strains show biocontrol activity against *N. ditissima* in apple shoots.

The selective recovery of rifampicin resistant bacterial colonies followed by ERIC-PCR genotyping was used to confirm the colonisation of each test bacterial strain in the detached apple stems. For example, this method identified that cross contamination for the shoots inoculated with *Bacillus* 42-1206(19)b$^{125ppmRif+}$ had occurred. Specific recovery of the inoculated mutant strain and confirmation by genotyping were also reported in previous studies (Andreote et al., 2010; Glandorf et al., 1992; Wicaksono et al., 2018; Wicaksono et al., 2017). A background bacterial strain was found to be resistant to 125 ppm rifampicin, as bacterial colonies recovered from the uninoculated control shoots and one shoot inoculated with 42-1206(19)$^{125ppmRif+}$ in the second colonisation assay were found to be identical based on the ERIC-PCR genotyping. The recovery of rifampicin-resistant background bacteria from untreated plants was not reported by previous studies (Andreote et al., 2010; Glandorf et al., 1992; Wicaksono et al., 2017). However, the number of rifampicin resistant background bacteria recovered in this study were very low. For the remaining treatments, the ERIC-PCR genotyping method verified that the bacteria recovered were those inoculated. In addition to the advantage in distinguishing the biocontrol candidates from

the background microflora (Bolstridge et al., 2009; Glandorf et al., 1992; Zinniel et al., 2002), the use of spontaneous antibiotic mutants enabled the confirmation that the inoculant bacteria remained viable which is difficult to achieve using PCR techniques because PCR amplifies DNA of both live and dead bacteria (Kloepper & Beauchamp, 1992).

The detection threshold value decreased from 3,000 CFU/cm in the first colonisation assay to 1,500 CFU/cm in the second colonisation assay allowing higher sensitivity in detecting the bacterial mutants in the stem tissue. Strains 20-579(18)$^{125ppmRif+}$ and 7-208(18)$^{125ppmRif+}$ were more effective at colonising the detached shoots than strain 31b3$^{125ppmRif+}$ in the second colonisation assay. A similar study was conducted in kiwifruit stem tissue by Wicaksono et al. (2018) who obtained data of population size with less variability compared to the current study. The possible reason for the variable data of population size in this study could be that apple stem tissue was difficult to macerate evenly so that the bacterial endophytes within the tissue were not effectively released from the stem tissue into the PBS (pH 7.2) used for dilution plating. Population size is another important criteria for selecting biocontrol candidates other than mechanism of action, because higher colonisation population may enhance biocontrol efficacy against *N. ditissima* in apple shoots. Enhancement of population size of biocontrol agents using nutritional amendments has been reported to be an effective way to enhance the biocontrol efficacy of biocontrol agents of fungal diseases (Ji & Wilson, 2003; Pagliaccia et al., 2008; Stanghellini & Miller, 1997; Yamada & Ogiso, 1997). The three *Pseudomonas* sp. strains which were shown to effectively colonise the apple stem tissue, with the recovered populations reaching 10^4 CFU/cm stem tissue were selected for the further study to determine their biocontrol activity in detached apple shoots.

No lesions developed around the point of inoculation and the leaf and shoots appeared healthy with no apparent symptoms, indicating that the strains used in this study were not pathogenic on apple. Similarly, Wicaksono et al. (2018) reported that the colonisation of kiwifruit stems by the inoculated endophytic bacteria did not cause any disease symptoms. All the strains used in the study were originally isolated from apparently healthy plants with the aim to limit the potential for isolating potential pathogens. However, McGrann and Brown (2017) reported that the fungal endophyte of barley, *Ramularia collo-cygni*, converted into a pathogen causing Ramularia leaf spot under abiotic stress associated with hydrogen peroxide levels in leaves. Therefore, further work is required to ensure that these endophytes have no potential to cause disease in apple tissues under natural conditions like in orchards or to check their potential pathogenicity by using a hypersensitivity test.

The results of the colonisation experiments in this Chapter revealed the potential of three of the bacterial endophyte strains to provide wound protection on apple shoots by persistent colonisation. The ability of the bacterial endophyte to spread from the inoculation site into the surrounding tissue also indicated the potential of these strains to suppress the growth of *N. ditissima* within the apple stem tissue. The endophytic bacterial strains identified were evaluated further for efficacy as biological control agents of *N. ditissima* in Chapter 5.

Chapter 5 Evaluation of endophytic bacteria for biocontrol of *Neonectria ditissima* infection of apple shoots

5.1 Introduction

As spores of *N. ditissma* are produced all year round (Ghasemkhani, 2012; Ohlendorf, 1999; Weber, 2014) and different wounds are also produced on apple trees throughout the year, there are frequent opportunities for infection (Alves & Nunes, 2017). Wound susceptibility is associated with wound type, wound size, inoculum dose, wound age, time of the year and cultivar (Alves & Nunes, 2017; Walter et al., 2016; Xu & Ridout, 1998). Picking wounds and leaf scars in autumn and pruning wounds are important entry sites for *N. ditissima* (Amponsah et al., 2015; Walter et al., 2019; Weber, 2014; Xu & Ridout, 1998). Leaf scars are susceptible up to 28 days after they are produced (Ohlendorf, 1999). Picking wounds are much bigger and more susceptible than leaf scars during the leaf fall period, though the number of picking wounds is lower than that of leaf scars. Also, picking wounds are difficult to be protected by fungicides as use of fungicides are more restricted during the fruit picking period than other times of the year (Amponsah et al., 2015). Susceptibility of pruning wounds was found to decrease as the age of wounds increase due to the wound healing (Xu & Ridout, 1998). Thus, to be effective in managing this threat, long-term and effective wound protection is required for biocontrol to be a viable option to control European canker development.

Control strategies currently used for protecting wounds are chemical-based fungicide sprays and wound paints (Anon., No date-c; Cooke, 1999; Xu & Butt, 1996). However, these are often not effective as it is difficult to protect all wounds produced, and repeat applications are required to provide year round protection. Recent work by Walter et al. (2019) reported that, although Captan was shown to be effective at reducing infection of leaf scar wounds by *N. ditissima*, it was not effective at protecting picking and rasp wounds from infection. Further, frequent use of chemical fungicides will increase the risk in the development of resistance in the pathogen. Biological products have been shown to reduce *N. ditissima* spore production from European canker lesions (Walter et al., 2017a). However, a range of biological products including Superzyme™ (*Bacillus subtilis, Pseudomonas putida, Trichoderma koningii* and *T. harzianum*), Clarity™ (*B. subtilis*), Fulzyme Plus™ (*B. subtilis*), µInoculant PP9^{A}™ (*P. putida*), Serenade™ Optimum (*B. subtilis*) and Vinevax™ (*T. harzianum*) applied as wound protectants did not provide effective control at high inoculum pressure (Walter et al., 2017b). Consequently, no biological products were recommended to be applied as a control strategy for European canker. Therefore, this study will focus on the use of endophytic bacteria, which can colonise the internal tissue, to

prevent infection of the wood tissue by *N. ditissima*. This strategy was successfully developed to reduce infection of grapevine shoots by the grapevine trunk pathogens *Neofusicoccum* spp. by using endophytic bacteria isolated from mānuka (*Leptospermum scoparium*) (Wicaksono et al., 2017).

The overall objective of this chapter was to determine the ability of the selected endophytic bacterial isolates to reduce *N. ditissima* infection of detached and attached apple shoots. The endophytic bacterial isolates that were shown to endophytically colonise detached apple shoots in Chapter 4 will be used here. The specific aims of the study were to (i) compare biocontrol activity of the wild type and mutant strains of the selected endophytic bacteria in detached shoots, (ii) identify biocontrol activity of bacterial mutants strains for long-term protection against *N. ditissima* in attached shoots and, iii) investigate the effect of timing of the endophytic bacterial inoculation in relation to the *N. ditissima* inoculation on infection.

5.2 Materials and methods

5.2.1 Pathogenicity of *N. ditissima* conidia in detached 'Royal Gala' shoots

To enable the ability of the endophytes to reduce *N. ditissima* infection and colonisation of shoot tissue to be assessed a robust infection protocol is required. The pathogenicity of different concentrations of a mixed isolate *N. ditissima* (isolates ICMP14417, MW15c1 and RS324p) conidia suspension produced on two different agars were compared on detached shoots of 'Royal Gala' in January 2018.

5.2.1.1 *Neonectria ditissima* conidia production

Two media, corn meal agar (CMA; BBL, Becton, Dickinson and Company, USA) and apple sap amended water agar (ASAWA) (Amponsah et al., 2014) were compared for their ability to support conidial production of *N. ditissima* isolates ICMP 14417, MW15c1 and RS324p using a modification of the method outlined by Amponsah et al. (2014). A 5-mm diameter mycelial disc was taken from the margin of a 2 to 3-week old colony of *N. ditissima* ICMP14417, MW15c1 or RS324p and placed in the centre of Petri dishes containing ASAWA or CMA. The plates were incubated at 20°C under a 16 h light (white fluorescent (PHILIPS TLD 36W BLB 1SL/25) lights) 8 h dark regime for 2-3 weeks.

After incubation, conidia were harvested as described by Scheper et al. (2014) from ASAWA or CMA plates. Conidia were washed from the colony by pipetting 2 mL sterile distilled water (SDW) containing 0.005% Tween 20 (Ajax Finechem, Thermo Fisher Scientific, Australia) 10 times over each culture plate with the same 2 mL used to wash conidia from four additional culture plates.

The number of conidia/ml in the suspension were then counted using a haemocytometer and adjusted to the required concentration using SDW.

In addition, the conidial viability as determined by percentage conidia germination was determined for each conidial suspension by placing six, 10 µL drops of a 10^4 conidia/mL suspension (10^2 conidia/drop) on PDA. The plates were incubated at 20°C in darkness for 4-6 h. After incubation, the percentage germination was assessed using a compound microscope at x 100 magnification. A conidium was considered to be germinated when the germ tube was at least the width of the conidium (Walter et al., 2016).

5.2.1.2 Plant material

The experiment was conducted using detached 'Royal Gala' shoots collected in early January 2018, summer in New Zealand. One-year old terminal shoots (approx. 25 cm long) collected from 'Royal Gala' trees in the Lincoln University Research Orchard were placed in water for 1 week before inoculation.

5.2.1.3 Inoculation method

A mixed *N. ditissima* isolate conidial suspension from conidia produced on ASAWA or CMA and adjusted to 10^5 conidia/mL and 10^4 conidia/mL were used as inocula. The mixed isolate conidial suspension was prepared by mixing equal volumes of 10^5 or 10^4 conidia/mL conidial suspension (Section 5.2.1.1) for each *N. ditissima* isolate. The conidial concentration in the final mixed isolates conidial suspension was confirmed as being 10^5 conidia/mL or 10^4 conidia/mL using a haemocytometer.

Ten µL of the mixed *N. ditissima* isolate conidial suspension at 10^5 conidia/mL (10^3 conidia/wound) or 10^4 conidia/mL (10^2 conidia/wound) were used to inoculate 3-mm wounds, as described in Section 4.2.6.3. Shoots serving as negative controls were inoculated with 10 µL of SDW plus 0.005% Tween 20 instead of the conidia suspension. The base of the shoots were placed in Universal bottles containing 20 mL water and liquid fertiliser (water: white Chrysal = 200: 1) and incubated on a light bench as described in the Section 4.2.6.3. Five replicates were set up for each treatment and assessment time, with the experiment arranged in a randomised block design with one replicate in each block.

5.2.1.4 Assessments

Plants were destructively harvested 14 and 28 days after inoculation to determine *N. ditissima* colonisation of the detached shoot at the point of inoculation (0 cm) and 1 cm, 2 cm, 3 cm, 4 cm and 5 cm above and below the inoculation point. Each shoot was surface sterilised and aseptically

dissected into 1 cm sections at each assessment point. Each 1-cm cut segment was cut into five pieces (1-2 mm thick) and plated on 1/5 strength of ASAWA. The plates were incubated at 20°C under a 16 h light (white fluorescent (PHILIPS TLD 36W BLB 1SL/25) lights) 8 h dark regime for 10 days after which colonisation of the stem pieces by *N. ditissima* was assessed. The identification of a representative number (90%) of colonies from stem pieces with white fluffy mycelia typical of *N. ditissima* were confirmed based on conidial morphology. To collect conidia produced from the stem pieces plated on the 1/5 strength of ASAWA plates, the method described by Monika Walter (Plant & Food Research, Motueka, NZ) was used. The base of a 1 mL pipette tip was cut (approx. 1 cm length) and placed into the agar surrounding a piece of stem tissue to be assessed to make a well (Figure 5.1a). Thirty µL of tap water was then added into the well and pipetted around five times to wash the conidia from the tissue. Samples of the resulting conidial suspension were then observed at x 400 magnification to determine the presence of *N. ditissima* conidia. The presence of *N. ditissima* conidia from at least one of the five tissue pieces per stem section was used to determine the presence of *N. ditissima* colonisation of the stem section.

5.2.2 Biocontrol activity of the wild type and mutants of the selected endophytic bacteria against *N. ditissima* in detached shoots of 'Royal Gala'

The three Rif^{125ppm} strains derived from the selected *Pseudomonas* spp. isolates which showed effective colonisation of the detached shoots (Sections 4.2.6 and 4.2.7) and their corresponding wild-type isolates were tested for their biocontrol activity against *N. ditissima* in detached shoots of 'Royal Gala' in January 2018.

5.2.2.1 Inoculation

Inoculum (10^6 CFU/mL) of the mutant and corresponding wild types of the three selected endophytic bacterial isolates were prepared using the method described in Section 4.2.6.2 and were then used to inoculate the detached shoots as described in Section 4.2.6.3 (10^4 cells/wound). The inoculated wounds (3 mm) were then covered by Parafilm™ 5 min after the inoculation. The base of the detached shoots were placed in Universal bottles containing 20 mL of water and liquid fertiliser (water: white Chrysal = 200: 1) and incubated on the light bench as described in Section 4.2.6.3.

Fourteen days after inoculation of the bacterial endophytes, the Parafilm™ was removed and a fresh wound was made at the same inoculation point on the shoot with a sterile scalpel. Ten microlitre of a 10^4 conidia/mL mixed isolate *N. ditissima* (isolates ICMP14417, MW15c1 and RS324p) conidial suspension produced on CMA as described in Section 5.2.1.1 was prepared using

the method described in Section 5.2.1.3 and inoculated on the fresh wound (10^2 conidia/wound). The new wounds were then covered by Parafilm™ again 5 min after inoculation.

Shoots inoculated with PBS (pH 7.2) (10 µL/wound) and followed by inoculation with SDW plus 0.005% Tween 20 (10 µL/wound) served as the negative control. Shoots inoculated with PBS (pH 7.2) (10 µL/wound) and followed by mixed isolate *N. ditissima* conidial suspension served as the positive control. Shoots inoculated with each bacterial endophyte and followed by inoculation with SDW plus 0.005% Tween 20 served as controls for endophytes, aiming at confirming the colonisation results of the detached shoot (Section 4.3.4), and that inoculation with the endophytes did not cause any disease symptoms on the shoots. Eight replicates were set up for each treatment and assessment time with the experiment arranged in a randomised block design with one replicate in each block.

5.2.2.2 Assessments

Plants were destructively harvested 14 and 28 days after *N. ditissima* inoculation to determine endophyte and *N. ditissima* colonisation of the detached shoot at the point of inoculation and 1 cm, 3 cm and 5 cm above and below the inoculation point. Each shoot was surface sterilised and aseptically dissected into 1 cm sections at each assessment point.

Eight replicate shoots were harvested after 14 days and 4 replicate shoots after 28 days to assess endophyte colonisation. For the shoots inoculated with the endophytic mutant strains followed by the inoculation with *N. ditissima* or SDW plus 0.005% Tween 20, each 1 cm section was sliced into six cross sectional pieces (approx. 1 mm thick) and three pieces plated equidistant on a 1/5 strength of ASAWA plate for recovery of *N. ditissima* and the remaining three pieces on an $NA^{Rif125+\ Cyclo50}$ (NA amended with 125 ppm Rifampicin and 50 ppm Cycloheximide) plate for recovery of the endophytic antibiotic resistant mutants. For shoots inoculated with wild type strains, each 1-cm segment were also cut into six pieces and 3 pieces randomly selected and plated on an ASAWA plate for recovery of *N. ditissima*. Randomly selected pieces were also plated on $NA^{Rif125+\ Cyclo50}$ in order to check for any rifampicin resistant background bacteria. Tissue pieces from the shoots inoculated with PBS followed by the inoculation of *N. ditissima* (positive control) or SDW plus 0.005% Tween 20 (negative control) were plated on the 1/5 strength of ASAWA.

For the 28 day after inoculation assessment, the four remaining replicate shoots were used to determine the endophyte population at the inoculation site. The 0.5 cm stem segment at the inoculation site was cut from the surface sterilised shoot and macerated using a sterile mortar and pestle in 2 mL of sterile PBS (pH 7.2), then serially diluted to 10 and 100-fold using PBS (pH

7.2). One-hundred microlitre aliquots of the 10^0, 10^{-1} and 10^{-2} tissue extract were spread plated on the $NA^{Rif125+\ Cyclo50}$. Duplicate plates were set up for each dilution level.

All the $NA^{Rif125+\ Cyclo50}$ plates were incubated at 25°C in the darkness for 1 week after which the presence of rifampicin-resistant bacterial colonies growing from the stem pieces were assessed, or the number of colonies were counted and used to determine the CFU/cm stem section at the inoculation site. The CFU data was $\log_{10}$ transformed and the $\log_{10}$ CFU/cm stem at the inoculation site for all the treatments were analysed by one-way ANOVA using Minitab 17. All the ASAWA plates were incubated at 20°C under a 16 h light (white fluorescent (PHILIPS TLD 36W BLB 1SL/25) lights) and 8 h dark regime in the incubator for 10 days and then assessed for colonisation of the tissue pieces by *N. ditissima* under a compound microscope as described in Section 5.2.1.4.

In addition, a representative number of the recovered bacterial isolates (n = ~12 from each bacterial mutant) were randomly selected from different treatments and different replicated shoots for genotypic identification using ERIC-PCR as described in the Section 4.2.4. The identity of any background rifampicin resistant isolates which grew from the negative control stem pieces were also confirmed by ERIC-PCR.

5.2.3 Biocontrol activity of the two selected bacterial mutants in attached shoots of 'Royal Gala' when inoculated before and after inoculation with *N. ditissima*.

Two Rif^{125ppm} strains derived from *Pseudomonas* spp. 20-579(18)b and 7-208(18)b were selected as inocula, because they were more effective at inhibiting the radial growth of *N. ditissima* isolates than $31b3^{125ppmRif+}$ (Section 4.3.2.2). Furthermore, selection of $7\text{-}208(18)b^{125ppmRif+}$ was also because the wild type 7-208(18)b was genetically more distant to both 20-579(18)b and 31b3, and potentially represented a different species (Figure 3.6).

'Royal Gala' potted trees (5 months old) grafted on rootstock 'M9' obtained from a commercial nursery (Waimea nursery, Nelson) were used for testing the biocontrol activity of the two endophyte mutant strains against *N. ditissima* in the glasshouse from March to July 2018. The plants were planted in 4 L pots containing potting mix composed of 20% pumice, 80% composted bark, 2 kg/m^3 Osmocote® Extract Standard 8-9 month gradual release fertilizer (16:3.5:10; N:P:K, respectively plus trace elements), 1 kg/m^3 agricultural lime, 500 g/m^3 Hydraflo® 2 (granular wetting agent, Scott Product New Zealand).

5.2.3.1 Inoculation

Inoculum for *Pseudomonas* spp. $20\text{-}579(18)b^{125ppmRif+}$ and $7\text{-}208(18)b^{125ppmRif+}$ (10^6 CFU/mL) and mixed *N. ditissima* isolates (10^4 conidia/mL) were produced and used to inoculate 3-mm wounds

made in the stem of the middle internode of the attached shoots as described in Section 5.2.2.1, except that two inoculation timings were set up. For the first, the shoots were inoculated with the endophyte mutant strain 14 days before the inoculation with *N. ditissima* as described in Section 5.2.2.1. For the second, the shoots were inoculated with the endophyte mutant strain 14 days after inoculation with *N. ditissima*. Seven replicates were set up for the treatments inoculated with both endophyte mutant strains and *N. ditissima* conidia, as well as the shoots inoculated with PBS and SDW plus 0.005% Tween 20 (negative control) and the shoots inoculated with PBS and *N. ditissima* conidia mixture (positive control). Four replicates were set up for the shoots inoculated with endophyte mutant strains and SDW plus 0.005% Tween 20 (endophyte controls), to confirm the colonisation results of the attached shoots (Section 4.3.4) and that inoculation with the endophytic bacteria did not cause any disease symptoms in the shoots. The experiment was arranged in a randomised block design in a glasshouse.

5.2.3.2 Assessment

The plants were destructively harvested 8 and 16 weeks after completion of all of the inoculation (either *N. ditissima* or endophyte). Before assessment of the endophyte and *N. ditissima* colonisation, all the shoots were observed for the development of symptoms typical of European canker at the two assessment times. Lesion length was measured using a digital calliper (Mitutoyo UK). Mean lesion lengths were analysed by one-way ANOVA using Minitab 17 followed by Fisher's LSD test.

Endophyte and *N. ditissima* colonisation of the stem tissue sections at the inoculation point and 1 cm, 3 cm, 5 cm, 7 cm and 9 cm above and below the inoculation point for all 7 replicates for the 8 week assessment were carried out using the method described in Section 5.2.2.2. For the 16 week assessment, the stem tissue sections 9 cm above and below the inoculation point each section were cut in half vertically with each half used to assess endophyte and *N. ditissima* colonisation, respectively. For the stem tissue section at the inoculation point at the 16 week assessment, one half was used for assessing *N. ditissima* colonisation, the other half was used for assessing the endophyte population by macerating the tissue section in 2 mL sterile PBS (pH 7.2) and spread plated on $NA^{Rif125+\ Cyclo50}$ (Section 5.2.2.2).

Plates were incubated and then assessed for endophyte and *N. ditissima* colonisation, respectively, as described in Section 5.2.2.2. Additionally, a representative number of the recovered bacterial isolates (n = ~12 from each bacterial mutant) were randomly selected for genotyping identification using ERIC-PCR as described in Section 4.2.4.

The threshold difference for defining a decrease or increase in the recovery frequency of *N. ditissima* around the inoculation point (+1 cm, 0 cm, -1 cm) in the attached shoots assay was ≥ 25%.

5.3 Results

5.3.1 Pathogenicity of *Neonectria ditissima* conidia in detached 'Royal Gala' shoots

Conidia were produced by colonies growing on both ASAWA and CMA for the three *N. ditissima* isolates ICMP14417, MW15c1 and RS324p. The conidial germination of the mixed isolate *N. ditissima* conidial suspension was ≥ 90% (data not shown) after 6 h on PDA at 20°C under white fluorescent lights.

No lesions developed on the detached shoots inoculated with the mixed isolate *N. ditissima* conidia suspension 28 days after inoculation in the pathogenicity test. However, white fluffy colonies typical of *N. ditissima* were reisolated from the shoots inoculated with the *N. ditissima* conidia (Figure 5.1a), but not from the uninoculated control shoots. From approximately 90% of the colonies, conidia identified as *N. ditissima* were microscopically observed (Figure 5.1b).

Colonisation of the detached shoots by *N. ditissima* is shown in Table 5.1. *Neonectria ditissima* was recovered from the inoculation site (0 cm) from apple shoots inoculated with conidia produced on ASAWA at both 10^4 and 10^5 conidia/mL at both 14 and 28 days after inoculation, on CMA at 10^4 conidia/mL at both 14 and 28 days after inoculation, and on CMA at 10^5 conidia/mL produced from CMA assessed after 28 days. It was not recovered from shoots inoculated with 10^5 conidia/mL produced from CMA assessed after 14 days. At the 28 days after inoculation, *N. ditissima* was recovered from apple shoots up to 4 cm above and 5 cm below the inoculation site when inoculated with 10^5 conidia/mL, and 4 cm above and 2 cm below the inoculation site when inoculated with 10^4 conidia/mL, when both inocula had been produced on CMA. At the same assessment time, *N. ditissima* was recovered from apple shoots up to 1 cm below and 2 cm above the inoculation site when inoculated with 10^4 conidia/mL and 10^5 conidia/mL produced on ASAWA. Based on the movement distance and colonisation at the two assessment times, 10^4 conidia/mL (10^2 conidia/wound) produced on CMA was selected as the *N. ditissima* inoculum in the following experiments. In general, recovery frequency of *N. ditissima* did not increase from 14 to 28 days (Table 5.1).

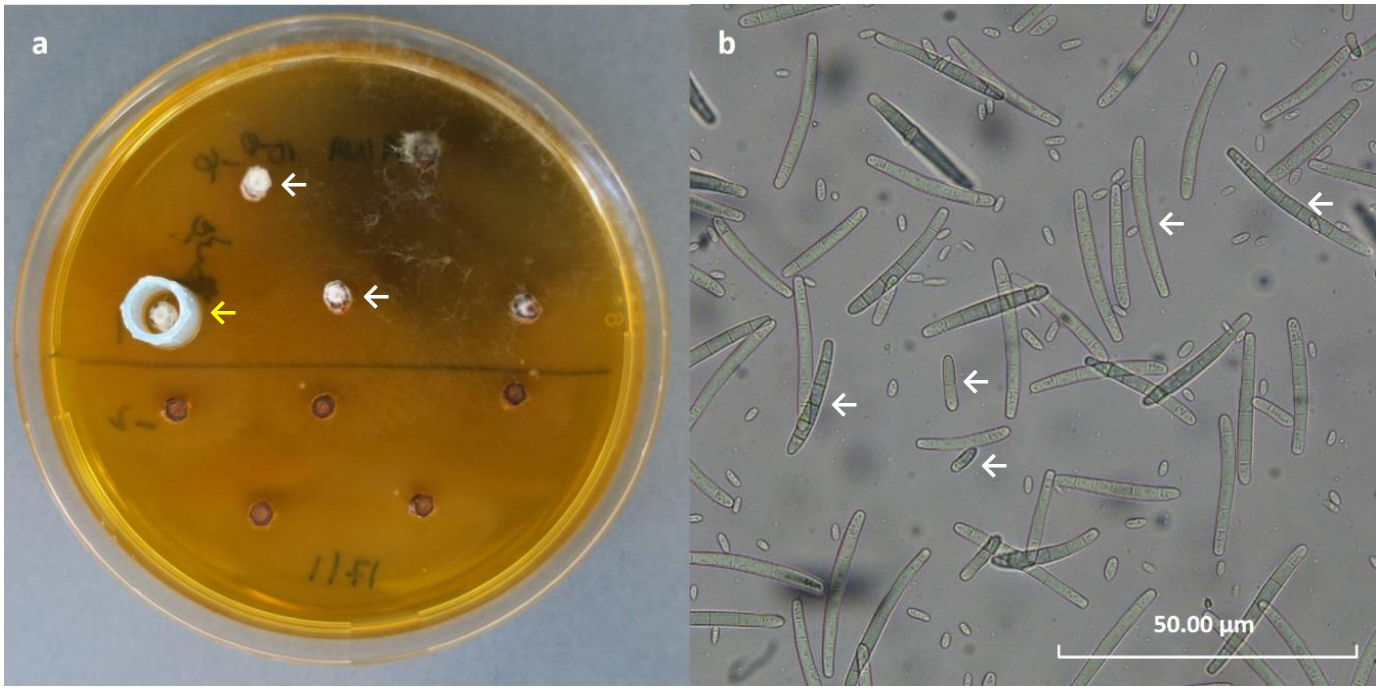

Figure 5.1 a) White fluffy mycelia typical of *N. ditissima* growing from stem pieces (indicated by white arrows) plated on 1/5 strength of apple sap amended water agar; cut base section of a 1 mL pipette tip (indicated by the yellow arrow) placed in the agar to produce a well for conidia collection. b) *Neonectria ditissima* conidia (indicated by white arrows) with 1, 2, 5 to 6 septate under a compound microscope at x 400 magnification.

Table 5.1 Percentage frequency of *Neonectria ditissima* recovered from 1-cm stem sections 5 cm above (+) and below (-) the inoculation point (0 cm) in five replicate detached apple shoots assessed 14 and 28 days after inoculation with *N. ditissima*. Shaded squares indicate stem sections where *N. ditissima* was isolated.

Agar for *N. ditissima* conidia production	Inoculation concentration (conidia/mL)	Days (d)	Percentage recovery of *N. ditissima* from stem tissue segments										
			+5 cm	+4 cm	+3 cm	+2 cm	+1 cm	0 cm	-1 cm	-2 cm	-3 cm	-4 cm	-5 cm
Negative control	Tween 20	14 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		28 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
ASAWA	10^4	14 d	0%	0%	0%	0%	0%	40%	20%	0%	0%	0%	0%
		28 d	0%	0%	0%	0%	0%	20%	20%	0%	0%	0%	0%
ASAWA	10^5	14 d	0%	0%	0%	0%	20%	20%	0%	0%	0%	0%	0%
		28 d	0%	0%	0%	20%	40%	40%	60%	0%	0%	0%	0%
CMA	10^4	14 d	0%	0%	0%	0%	40%	60%	0%	0%	0%	0%	0%
		28 d	0%	40%	0%	0%	40%	40%	20%	20%	0%	0%	0%
CMA	10^5	14 d	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		28 d	0%	20%	0%	40%	40%	20%	0%	40%	20%	20%	20%

5.3.2 Comparison of biocontrol activity between the wild-type isolates and mutant strains of the three selected endophytic bacteria in detached 'Royal Gala' shoots

The percentage germination of the mixed isolate *N. ditissima* (ICMP14417, MW15c1 and RS324p) conidia suspension used in the experiment was ≥90% (data not shown). No European canker symptoms or lesions were observed on any of the shoots inoculated with *N. ditissima*, including the shoots serving as positive controls, 14 days and 28 days after *N. ditissima* inoculation. In the endophyte control shoots (inoculation of endophyte mutant strains and SDW plus 0.005% Tween 20), no lesions developed around the point of inoculation and the shoots appeared healthy with no apparent symptoms.

No rifampicin resistant colonies were recovered from the shoots inoculated with PBS (pH 7.2) (negative control and *N. ditissima* positive control) at either of the two assessment times (Table 5.2). No resistant bacterial colonies were recovered from the stem pieces of a representative number of shoots (n = 10 at each time assessment) inoculated with the wild type strains of the tested bacteria (data not shown). In general, rifampicin resistant bacterial colonies were recovered from the stem sections up to 5 cm above and below the inoculation point from the apple shoots inoculated with all the three bacterial mutant strains at both assessment times (Table 5.2). Typically, recovery frequency of the endophyte mutant strains decreased from the stem sections further from the inoculation point (Table 5.2). Inoculation with *N. ditissima* did not change the colonisation of the shoot by the bacterial strains.

A representative number (n = ~12) of the rifampicin resistant bacterial colonies recovered from shoots inoculated with each endophyte mutant strain were verified by ERIC-PCR to be the inoculated strains (Figure 5.2). Two rifampicin resistant bacterial colonies recovered from shoots inoculated with a wild type endophyte showed the same genotype as 7-208(18)b. However, which wild type the shoot was inoculated with was not recorded. One rifampicin resistant bacterial colony recovered from shoots inoculated with PBS showed the same genotype as 20-579(18)b, while the other two rifampicin resistant bacterial colonies recovered from shoots inoculated with PBS showed different genotypes from any of the inoculated strains.

Table 5.2 Percentage frequency of rifampicin-resistant bacterial colonies recovered from 1 cm stem segments above (+) and below (-) the inoculation wound (0 cm) in eight replicate detached apple shoots assessed 14 days after inoculation of bacterial endophyte mutants/PBS and *Neonectria ditissima*/0.005% Tween 20 and four replicate shoots assessed 28 days after inoculation of bacterial endophyte mutants/PBS and *Neonectria ditissima*/0.005% Tween 20. Shaded squares indicate stem sections where rifampicin-resistant bacteria were isolated.

Treatments		Days (d)	Percentage recovery of resistant bacterial colonies from stem tissue segments						
Endophyte	*N. ditissima*		+5 cm	+3 cm	+1 cm	0 cm	-1 cm	-3 cm	-5 cm
PBS (negative control)	Tween 20	14 d	0%	0%	0%	0%	0%	0%	0%
		28 d	0%	0%	0%	0%	0%	0%	0%
PBS (positive control)	*N. ditissima*	14 d	0%	0%	0%	0%	0%	0%	0%
		28 d	0%	0%	0%	0%	0%	0%	0%
20-579(18)b$^{125ppmRif+}$	Tween 20	14 d	63%	50%	88%	100%	100%	88%	75%
		28 d	50%	100%	100%	100%	100%	100%	100%
20-579(18)b$^{125ppmRif+}$	*N. ditissima*	14 d	75%	88%	88%	100%	100%	88%	100%
		28 d	50%	75%	75%	100%	100%	100%	75%
7-208(18)b$^{125ppmRif+}$	Tween 20	14 d	63%	75%	100%	100%	100%	100%	100%
		28 d	0%	75%	100%	100%	100%	100%	100%
7-208(18)b$^{125ppmRif+}$	*N. ditissima*	14 d	63%	75%	100%	100%	100%	100%	88%
		28 d	75%	100%	100%	100%	75%	100%	75%
31b3$^{125ppmRif+}$	Tween 20	14 d	100%	100%	100%	100%	100%	100%	75%
		28 d	50%	50%	100%	100%	100%	75%	67%
31b3$^{125ppmRif+}$	*N. ditissima*	14 d	100%	88%	100%	100%	100%	88%	75%
		28 d	50%	100%	100%	100%	75%	75%	100%

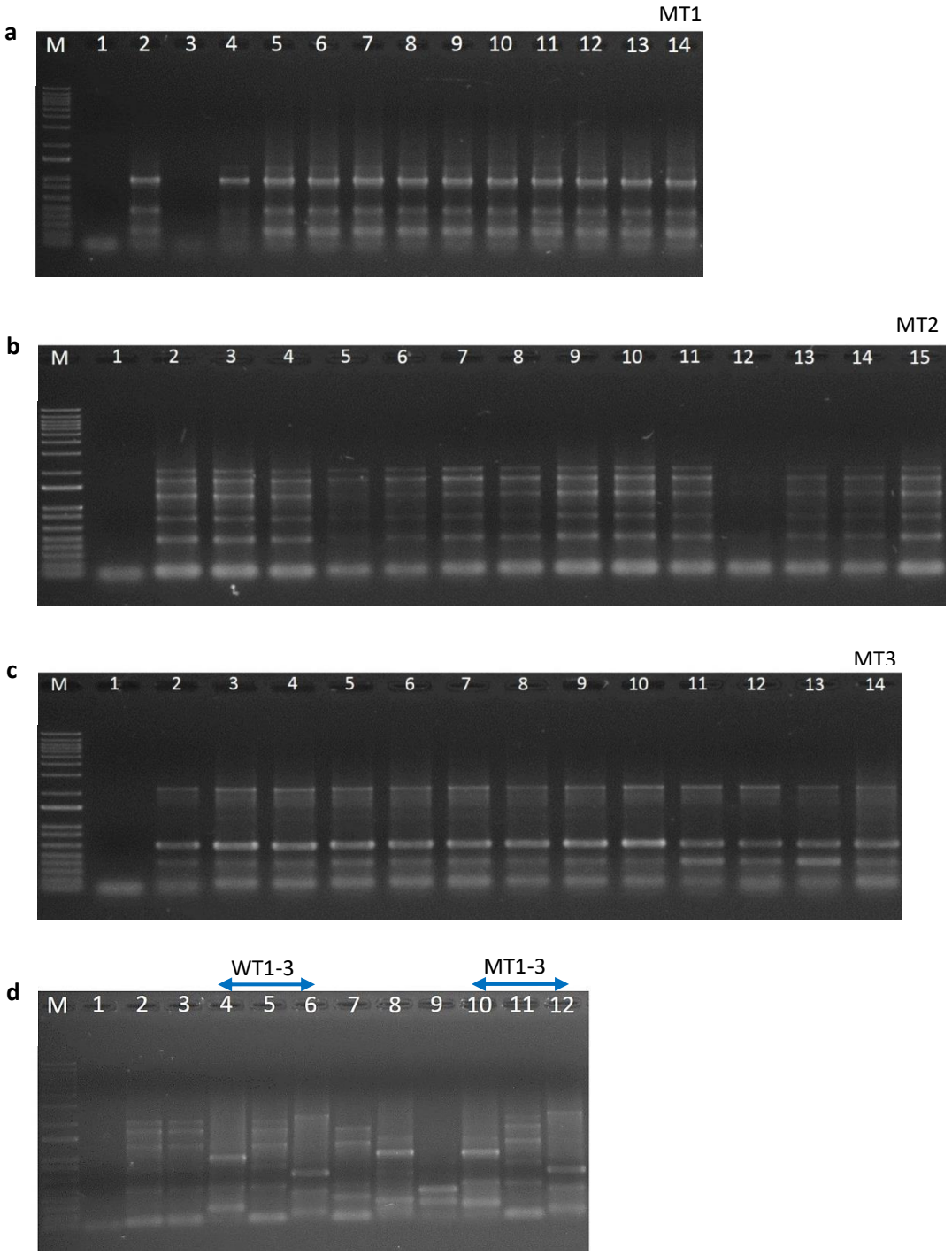

Figure 5.2 Agarose gel showing genotypes of the bacterial colonies recovered from the apple shoots inoculated with the wildtype strains, rifampicin-resistant mutants, or PBS compared with that of a pure culture of the inocula. M: 1 kb plus ladder (Invitrogen), 1: negative control (sterile PCR water). a) 2-13: bacterial colonies recovered from the shoots inoculated with 20-579(18)$^{125ppmRif+}$, 14 (MT1): 20-579(18)$^{125ppmRif+}$; b) 2-14: bacterial colonies recovered from the shoots inoculated with 7-208(18)$^{125ppmRif+}$, 15 (MT2): 7-208(18)$^{125ppmRif+}$; c) 2-13: bacterial colonies recovered from the shoots inoculated with 31b3$^{125ppmRif+}$, 14 (MT3): 31b3$^{125ppmRif+}$; d) 2, 3: bacterial colonies recovered from shoots inoculated with an wildtype endophyte; 4-6 (WT1-3): 20-579(18)b, 7-208(18)b and 31b3; 7-9: background rifampicin resistant bacteria recovered from shoots inoculated with PBS; 10-12: 20-579(18)$^{125ppmRif+}$,7-208(18)$^{125ppmRif+}$ and 31b3$^{125ppmRif+}$.

There was no significant difference (p = 0.104, Appendix A5.1) in the population of the three tested bacterial mutants in the shoots at the inoculation point for shoots inoculated with *N. ditissima* or uninoculated assessed 28 days after inoculation, with population size ranging from 3.76 to 4.99 $\log_{10}$ CFU/cm stem section (Table 5.3).

Table 5.3 Mean population of rifampicin-resistant bacterial mutants ($\log_{10}$ CFU/cm stem) recovered from the inoculation point of detached apple shoots (four replicates) inoculated with the three tested bacterial mutants assessed 28 days after inoculation with *Neonectria ditissima* (+) or 0.005% Tween 20 as untreated control (-).

Inoculation treatment		Mean population of bacterial mutants ($\log_{10}$ CFU/cm stem)
Bacterial endophyte	*N. ditissima*	
20-579(18)b$^{125ppmRif+}$	-	4.360
20-579(18)b$^{125ppmRif+}$	+	4.607
7-208(18)b$^{125ppmRif+}$	-	4.987
7-208(18)b$^{125ppmRif+}$	+	4.710
31b3$^{125ppmRif+}$	-	3.761
31b3$^{125ppmRif+}$	+	4.410
p value		0.104

The detection threshold value is 1,200 CFU/cm stem (3.079 $\log_{10}$ CFU/cm stem section).

N. ditissima was not recovered from the negative control shoots (no *N. ditissima* inoculation) at either of the two assessment times (Table 5.4), indicating that there was no background infection by *N. ditissima*. For each assessed 1-cm stem segment, 1-2 stem pieces with white fluffy mycelia typical of *N. ditissima* growing were verified to be *N. ditissima* using a compound microscope (data not shown). *N. ditissima* was recovered from the shoot up to 5 cm above the inoculation point for the positive control (PBS + *N. ditissima*) 28 days after inoculation with *N. ditissima* (Table 5.4). Inoculation with the wild type or mutant strains did not reduce the recovery of *N. ditissima* from the shoots compared with the *N. ditissima* positive control. *N. ditissima* recovery was similar between shoots inoculated with wild type strains and shoots inoculated with mutant strains (Table 5.4). In all the treatments inoculated with *N. ditissima*, recovery frequency of *N. ditissima* decreased from the stem sections further from the inoculation point at both assessment times (Table 5.4).

Table 5.4 Percentage frequency of *Neonectria ditissima* recovered from 1 cm stem sections above (+) and below (-) the inoculation point (0 cm) in detached apple shoots assessed 14 and 28 days after inoculation with 3 bacterial endophyte mutant strains (MT) or their corresponding wildtype (WT) or PBS followed by *N. ditissima* or Tween 20 (0.005%). Shaded squares indicate stem sections where *N. ditissima* were isolated.

Treatment			Percentage recovery of *N. ditissima* from stem tissue segments													
			+5 cm		+3 cm		+1 cm		0 cm		-1 cm		-3 cm		-5 cm	
Endophyte	*N. ditissima*		14 d	28 d	14 d	28 d	14 d	28 d	14 d	28 d	14 d	28 d	14 d	28 d	14 d	28 d
PBS	Tween 20	NC	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
20-579(18)b	Tween 20	WT	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		MT	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
7-208(18)b	Tween 20	WT	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		MT	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
31b3	Tween 20	WT	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		MT	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
PBS	*N. ditissima*	PC	0%	25%	0%	0%	38%	50%	88%	75%	63%	25%	0%	25%	0%	0%
20-579(18)b	*N. ditissima*	WT	0%	0%	0%	0%	50%	50%	100%	100%	33%	50%	0%	0%	0%	25%
		MT	0%	0%	0%	0%	13%	25%	88%	100%	50%	50%	0%	25%	0%	0%
7-208(18)b	*N. ditissima*	WT	0%	0%	0%	0%	67%	25%	100%	100%	33%	25%	0%	0%	17%	0%
		MT	0%	0%	0%	0%	25%	50%	75%	100%	13%	0%	0%	25%	0%	0%
31b3	*N. ditissima*	WT	0%	0%	0%	0%	50%	100%	100%	100%	50%	100%	0%	0%	0%	0%
		MT	0%	0%	0%	25%	63%	50%	88%	75%	63%	25%	0%	0%	0%	0%

'NC' and 'PC' mean negative control and positive control, respectively. There were eight replicates shoots for the bacterial mutant (MT) assessed 14 days after inoculation and four replicates assessed 28 days after inoculation. There were six replicates for the wild type of bacterial isolates (WT) assessed 14 days after inoculation and four replicates assessed 28 days after inoculation.

5.3.3 Biocontrol activity of the two selected bacteria mutants in attached shoots of 'Royal Gala' when inoculated before and after the inoculation of *N. ditissima*

Although no reduction in *N. ditissima* colonisation by the three rifampicin resistant mutant strains in the detached shoots was observed, the experiment to determine the effect of inoculation with *Pseudomonas* sp. strains 20-579(18)b$^{125ppmRif+}$ and 7-208(18)b$^{125ppmRif+}$ on *N. ditissima* colonisation in attached shoots was carried out.

In the endophyte control treatments (inoculation of endophyte mutant strains before or after the inoculation of SDW plus 0.005% Tween 20), no lesions developed around the point of inoculation and the plants appeared healthy with no apparent symptoms.

Recovery frequency of rifampicin-resistant bacterial colonies from the stem section for the different treatments are shown in Table 5.5. No rifampicin resistant bacterial colonies were recovered from the positive and negative control shoots inoculated with PBS (no endophyte mutants) at either assessment time. At the 8 week assessment, rifampicin resistant bacterial colonies were recovered from the stem sections up to 7-9 cm above/below the inoculation point from the apple shoots inoculated with *Pseudomonas* sp. isolates 20-579(18)b$^{125ppmRif+}$ and 7-208(18)b$^{125ppmRif+}$, irrespective of inoculation of *N. ditissima* or Tween 20 before or after the inoculation of endophyte (Table 5.5). At the 16 week assessment, for both endophyte mutant strains, rifampicin resistant bacterial colonies were recovered from stem sections further from the inoculation point for shoots inoculated with *N. ditissima* before or after the inoculation of the endophytes, as compared to those inoculated with Tween 20 instead (Table 5.5). Also, at the 16 weeks assessment for *Pseudomonas* sp. isolate 7-208(18)b$^{125ppmRif+}$, there was an indication of higher colonisation frequency of the stem pieces when the endophyte was inoculated 14 days after inoculation with *N. ditissima* in all the assessed stem tissue segments, as compared with those shoots inoculated with Tween 20 instead of *N. ditissima*. This pattern was not obvious for other treatments including the inoculation of endophytes before or after the inoculation of *N. ditissima*. At both of the two assessment times, colonisation frequency and movement of the two endophytic bacterial mutants was generally not affected by the inoculation of *N. ditissima* inoculated 14 days before or after. A representative number (n = ~12) of the rifampicin resistant bacterial colonies were verified by ERIC-PCR to be the inoculating strains (Figure 5.3).

Table 5.5 Percentage frequency of rifampicin-resistant bacterial colonies recovered from 1 cm stem segments above (+) and below (-) the inoculation wound (0 cm) in apple shoots assessed 8 and 16 weeks after inoculation with endophytic *Pseudomonas* sp. mutant strains/PBS followed 14 days later with *Neonectria ditissima*/0.005% Tween 20, or *N. ditissima*/0.005% Tween 20 followed 14 days later with endophytic *Pseudomonas* sp. mutant strains/PBS. Shaded squares indicate stem sections where the rifampicin-resistant bacteria were isolated.

Treatments		Rep	Weeks	Percentage recovery of resistant bacterial colonies from stem tissue segments										
Initial	14 days later			+9 cm	+7 cm	+5 cm	+3 cm	+1 cm	0 cm	-1 cm	-3 cm	-5 cm	-7 cm	-9 cm
PBS	Tween 20 (NC)	7	8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
			16	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
PBS	*N. ditissima* (PC)	7	8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
			16	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
20-579(18)b$^{125ppmRif+}$	Tween 20	4	8	0%	25%	50%	100%	100%	100%	100%	75%	0%	50%	0%
			16	0%	50%	50%	25%	100%	100%	75%	25%	25%	0%	0%
20-579(18)b$^{125ppmRif+}$	*N. ditissima*	7	8	0%	0%	14%	29%	100%	100%	100%	57%	29%	29%	14%
			16	14%	14%	43%	86%	86%	100%	100%	57%	29%	14%	14%
7-208(18)b$^{125ppmRif+}$	Tween 20	4	8	25%	50%	75%	75%	100%	100%	100%	75%	50%	50%	0%
			16	0%	0%	25%	100%	100%	100%	75%	75%	50%	0%	0%
7-208(18)b$^{125ppmRif+}$	*N. ditissima*	7	8	14%	29%	43%	57%	71%	100%	100%	43%	14%	0%	0%
			16	0%	0%	43%	43%	100%	71%	100%	71%	29%	14%	14%
Tween 20	PBS (NC)	7	8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
			16	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
N. ditissima	PBS (PC)	7	8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Table 5.5 Continued

Treatments		Rep	Weeks	Percentage recovery of resistant bacterial colonies from stem tissue segments										
Initial	14 days later			+9 cm	+7 cm	+5 cm	+3 cm	+1 cm	0 cm	-1 cm	-3 cm	-5 cm	-7 cm	-9 cm
			16	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Tween 20	20-579(18)b$^{125ppmRif+}$	4	8	25%	25%	25%	50%	100%	100%	100%	50%	25%	25%	50%
			16	0%	0%	0%	25%	100%	75%	75%	25%	0%	0%	0%
N. ditissima	20-579(18)b$^{125ppmRif+}$	7	8	0%	0%	14%	43%	100%	100%	86%	57%	57%	43%	14%
			16	0%	14%	14%	57%	100%	100%	100%	57%	14%	14%	14%
Tween 20	7-208(18)b$^{125ppmRif+}$	4	8	25%	25%	50%	75%	50%	100%	100%	25%	25%	25%	25%
			16	0%	0%	0%	50%	50%	50%	50%	25%	50%	0%	0%
N. ditissima	7-208(18)b$^{125ppmRif+}$	7	8	0%	29%	86%	86%	100%	100%	100%	86%	43%	29%	29%
			16	14%	29%	43%	71%	71%	71%	86%	86%	57%	14%	14%

'NC' and 'PC' refer to negative control and positive control, respectively. At the 16 week assessment, half of the 1 cm stem segments at the inoculation point were used to assess for endophyte mutant and *N. ditissima* recovery. The other half of the stem tissue was used for assessing endophyte mutant population shown in the Table 5.6.

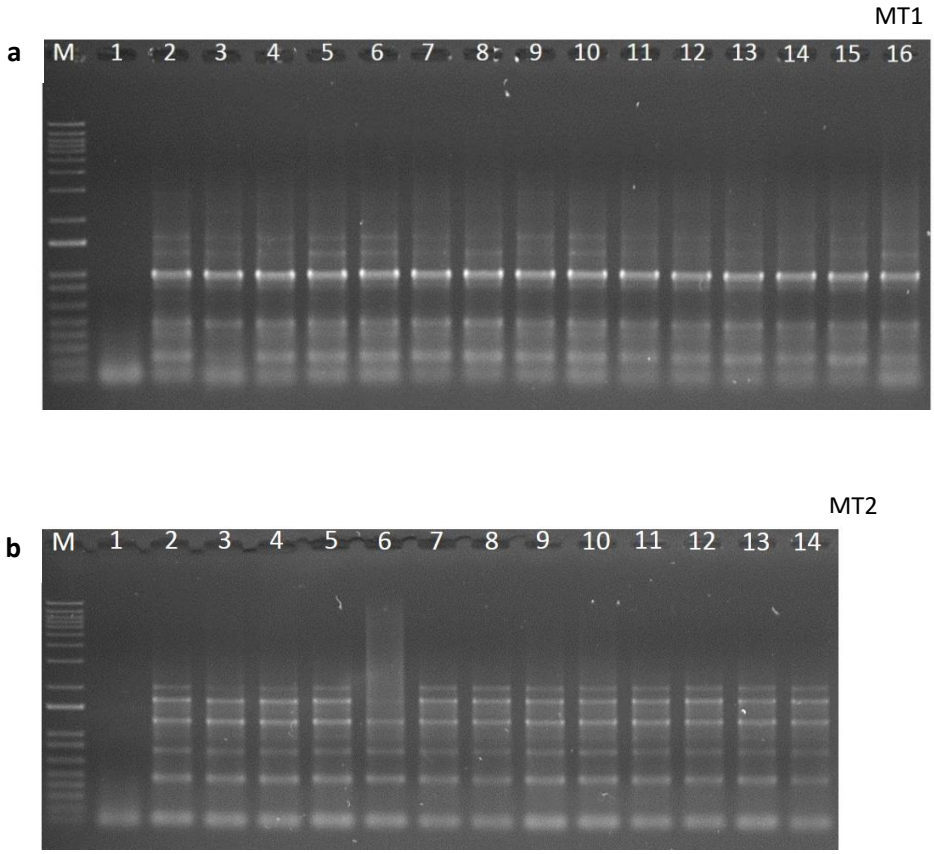

Figure 5.3 Agarose gel showing genotypes of the bacterial colonies recovered from the apple shoots inoculated with rifampicin-resistant mutants compared with that of a pure culture of the inocula. M: 1 kb plus ladder (Invitrogen), 1: negative control (sterile PCR water). a) 2-15: bacterial colonies recovered from the shoots inoculated with 20-579(18)$^{125ppmRif+}$, 16 (MT1): 20-579(18)$^{125ppmRif+}$; b) 2-13: bacterial colonies recovered from the shoots inoculated with 7-208(18)$^{125ppmRif+}$, 14 (MT2): 7-208(18)$^{125ppmRif+}$.

There was no significant difference (p = 0.846, Appendix A5.2) in the population of rifampicin resistant colonies recovered from the stem section at the inoculation point for shoots inoculated with the two *Pseudomonas* sp. mutant strains before or after inoculation with *N. ditissima*/Tween 20 assessed 16 weeks after the inoculation (Table 5.6).

Table 5.6 Mean population of rifampicin-resistant bacteria (log_{10} CFU/ half of 1 cm stem section) recovered from half of the 1 cm stem section at the inoculation point of attached apple shoots inoculated with two *Pseudomonas* sp. mutant strains at the 16 weeks assessment. Shoots were inoculated with endophytic *Pseudomonas* sp. mutant strains followed 14 days later with *Neonectria ditissima*/0.005% Tween 20 or *N. ditissima*/0.005% Tween 20 followed 14 days later with endophytic *Pseudomonas* sp. mutant strains.

Treatments		Mean population of bacterial mutants
Initial	14 days later	(log_{10} CFU/half of 1 cm stem)
20-579(18)b$^{125ppmRif+}$	Tween 20	4.068
20-579(18)b$^{125ppmRif+}$	*N. ditissima*	3.844
7-208(18)b$^{125ppmRif+}$	Tween 20	4.444
7-208(18)b$^{125ppmRif+}$	*N. ditissima*	3.714
Tween 20	20-579(18)b$^{125ppmRif+}$	3.478
N. ditissima	20-579(18)b$^{125ppmRif+}$	3.662
Tween 20	7-208(18)b$^{125ppmRif+}$	4.225
N. ditissima	7-208(18)b$^{125ppmRif+}$	4.041
p value		0.846

The detection threshold is 600 CFU/half of 1 cm stem piece (2.778 log_{10} CFU/half of 1 cm stem).

Recovery frequency of *N. ditissima* from each treatment is shown in Table 5.7. *N. ditissima* was not recovered from any of the shoots which were not inoculated with *N. ditissima* at either of the two assessment times indicating that there was no background infection by *N. ditissima*. For each assessed 1-cm stem segment, 1-2 stem pieces with white fluffy mycelia typical of *N. ditissima* growing were observed by a compound microscope and verified to be *N. ditissima* (data not shown).

At the 8 weeks assessment, *N. ditissima* was recovered from 1 cm above and 3 cm below the inoculation point in the shoots inoculated with *Pseudomonas* sp. isolate 20-579(18)b$^{125ppmRif+}$ followed by *N. ditissima.* This was shorter than the length of shoot from which *N. ditissima* was recovered in the positive control (PBS followed by *N. ditissima*) treatment being recovered at 9 cm above and 5 cm below the inoculation point. The recovery frequency of *N. ditissima* from the stem sections 1 cm above the inoculation point was decreased by 57%, respectively for the *Pseudomonas* sp. isolate 20-579(18)b$^{125ppmRif+}$compared with the *N*. *ditissima* positive control. At the 16 weeks assessment, the movement of *N. ditissima* was similar in these two treatments, with reduction in the percentage recovery of *N. ditissima* from the stem sections 1 cm above the inoculation point of 57%, respectively as compared to the *N. ditissima* positive control (Table 5.7).

For shoots inoculated with *Pseudomonas* sp. isolate 7-208(18)b$^{125ppmRif+}$ followed by *N. ditissima*, there was generally no reduction in recovery frequency of *N. ditissima* compared with the positive control (PBS followed by *N. ditissima*) shoots at each assessed stem segment at the 8 weeks assessment. However, at the 16 weeks assessment the recovery frequency of *N. ditissima*

was lower than the positive control from the stem sections at the inoculation point (reduced by 43%), 1 cm above the inoculation point (reduced by 42%) and 1 cm below the inoculation point (reduced by 28%). The movement of *N. ditissima* was not restricted by the introduction of 7-208(18)b$^{125ppmRif+}$ at either assessment times (Table 5.7).

In the treatments where *N. ditissima* was inoculated 14 days prior to the *Pseudomonas* sp. mutant strains, at the 8 weeks assessment *N. ditissima* was recovered from stem sections 5 cm above and below the inoculation point in both of the treatments inoculated with *Pseudomonas* sp. isolates 20-579(18)b$^{125ppmRif+}$ and 7-208(18)b$^{125ppmRif+}$. This was comparable to the length of tissue colonised by *N. ditissima* in the positive control shoots (*N. ditissima* followed by PBS). At the 16 weeks assessment, *N. ditissima* was recovered from 9 cm above and below the inoculation point in the shoots inoculated with *Pseudomonas* sp. isolate 20-579(18)b$^{125ppmRif+}$, and 9 cm above and 7 cm below the inoculation point in the shoots inoculated with *Pseudomonas* sp. isolate 7-208(18)b$^{125ppmRif+}$. This was further than the positive control (5 cm above and 3 cm below the inoculation point). At both assessments, the recovery frequency of *N. ditissima* was higher in the stem sections around the inoculation point (inoculation point, 1 cm above and below the inoculation point) in the shoots inoculated with the two *Pseudomonas* sp. mutant strains compared with the positive control (*N. ditissima* followed by PBS). At the 8 weeks assessment, the recovery frequency of *N. ditissima* was increased by 33% at 1 cm above the inoculation point in the shoots inoculated with 20-579(18)b$^{125ppmRif+}$, and by 33% at 1 cm below the inoculation point in the shoots inoculated with 7-208(18)b$^{125ppmRif+}$. This was more obvious at the 16 weeks assessment, the recovery frequency of *N. ditissima* increased by 43%-71% around the inoculation point in the shoots inoculated with 20-579(18)b$^{125ppmRif+}$, and increased by 29%-43% around the inoculation point in the shoots inoculated with 7-208(18)b$^{125ppmRif+}$.

Table 5.7 Percentage frequency of *Neonectria ditissima* recovered from 1 cm stem sections above (+) and below (-) the inoculation point (0 cm) in attached apple shoots assessed 8 and 16 weeks after inoculation with endophytic *Pseudomonas* sp. mutant strains/PBS followed 14 days later with *Neonectria ditissima*/0.005% Tween 20, or *N. ditissima*/0.005% Tween 20 followed 14 days later with endophytic *Pseudomonas* sp. mutant strains/PBS. Shaded squares indicate stem sections where *N. ditissima* was isolated.

Treatments		Rep	Weeks	Percentage recovery of *N. ditissima* colonies from stem tissue segments										
Initial	14 days later			+9 cm	+7 cm	+5 cm	+3 cm	+1 cm	0 cm	-1 cm	-3 cm	-5 cm	-7 cm	-9 cm
PBS	Tween 20 (NC)	7	8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
			16	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
PBS	*N. ditissima* (PC)	7	8	14%	0%	14%	0%	86%	86%	57%	0%	14%	0%	0%
			16	0%	0%	14%	29%	71%	57%	57%	14%	0%	0%	0%
20-579(18)b$^{125ppmRif+}$	Tween 20	4	8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
			16	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
20-579(18)b$^{125ppmRif+}$	*N. ditissima*	7	8	0%	0%	0%	0%	29%	100%	43%	14%	0%	0%	0%
			16	0%	0%	14%	14%	14%	57%	43%	14%	0%	0%	0%
7-208(18)b$^{125ppmRif+}$	Tween 20	4	8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
			16	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
7-208(18)b$^{125ppmRif+}$	*N. ditissima*	7	8	0%	14%	14%	57%	71%	86%	57%	57%	29%	14%	0%
			16	0%	14%	14%	29%	29%	14%	29%	0%	0%	0%	0%
Tween 20	PBS (NC)	7	8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
			16	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
N. ditissima	PBS (PC)	7	8	0%	0%	17%	67%	67%	83%	67%	33%	17%	0%	0%
			16	0%	0%	14%	43%	43%	57%	29%	43%	0%	0%	0%

Table 5.7 continued

Treatments		Rep	Weeks	Percentage recovery of *N. ditissima* colonies from stem tissue segments										
Initial	14 days later			+9 cm	+7 cm	+5 cm	+3 cm	+1 cm	0 cm	-1 cm	-3 cm	-5 cm	-7 cm	-9 cm
Tween 20	20-579(18)b$^{125ppmRif+}$	4	8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
			16	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
N. ditissima	20-579(18)b$^{125ppmRif+}$	7	8	0%	0%	14%	57%	100%	100%	71%	29%	14%	0%	0%
			16	57%	43%	43%	71%	86%	100%	100%	43%	14%	29%	14%
Tween 20	7-208(18)b$^{125ppmRif+}$	4	8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
			16	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
N. ditissima	7-208(18)b$^{125ppmRif+}$	7	8	0%	0%	14%	57%	86%	86%	100%	71%	29%	0%	0%
			16	57%	57%	57%	71%	86%	86%	71%	57%	14%	14%	0%

'NC' and 'PC' refer to negative control and positive control, respectively. The percentage data in green showed reduced recovery frequency of *N. ditissima* by treatment with endophyte mutant strains as compared with the positive control (PBS followed by *N. ditissima*). The percentage data in red showed increased recovery frequency of *N. ditissima* by treatment with endophyte mutant strains as compared with the positive control (*N. ditissima* followed by PBS). At the 16 weeks assessment, half of the 1 cm stem segments at the inoculation point were used to assess for endophyte mutant and *N. ditissima* recovery. The other half of the stem tissue was used for assessing endophyte mutant population shown in the Table 5.6. The threshold difference for defining a decrease or increase in the recovery frequency of *N. ditissima* around the inoculation point (+1 cm, 0 cm, -1 cm) was ≥ 25%.

No lesions were observed for any of the *N. ditissima* inoculated treatment 4 weeks after inoculation (data not shown).

At the 8 weeks assessment, there was a significant effect (p = 0.026, Appendix A5.3) of treatment on lesion length. The lesion length in the shoots inoculated with *Pseudomonas* sp. isolate 7-208(18)b$^{125ppmRif+}$ before *N. ditissima* was significantly shorter than the lesion length in shoots inoculated with *N. ditissima* before the two endophytic *Pseudomonas* sp. isolates (Table 5.8). However, there was no significant difference in the lesion length between the treatments inoculated with either of the two endophytic *Pseudomonas* sp. isolates before *N. ditissima* and the positive control (PBS followed by *N. ditissima*). At the 16 weeks assessment, there was no significant effect (p = 0.819, Appendix A5.4) of treatment on lesion length (Table 5.8). No European canker symptoms or lesions developed on any of the shoots uninoculated with *N. ditissima* at both assessment times. Flaky bark, a typical European canker symptom, was observed surrounding almost all the lesions at the inoculation point of the shoots inoculated with *N. ditissima*. Representative pictures of shoots with and without lesion development are shown in Figure 5.4.

Table 5.8 Mean of lesion length on the shoots with *Neonectria ditissima* inoculated measured at the 8 weeks and 16 weeks shoot harvesting time. Shoots were inoculated with endophytic *Pseudomonas* sp. mutant strains/PBS followed 14 days later with *N. ditissima*/Tween 20 or *N. ditissima*/Tween 20 followed 14 days later with endophytic *Pseudomonas* sp. mutant strains/PBS.

Treatment		Mean of lesion length (mm)	
Initial	14 days later	8 weeks	16 weeks
PBS	*N. ditissima* (positive control)	14.09 ab	16.06
20-579(18)b$^{125ppmRif+}$	*N. ditissima*	13.54 ab	16.06
7-208(18)b$^{125ppmRif+}$	*N. ditissima*	10.23 b	17.70
N. ditissima	PBS (positive control)	18.29 a	15.87
N. ditissima	20-579(18)b$^{125ppmRif+}$	18.05 a	16.25
N. ditissima	7-208(18)b$^{125ppmRif+}$	18.00 a	13.45
p value		0.026*	0.819

Means followed by the same letter within a column are not significantly different. * significantly different ($p \leq 0.05$).

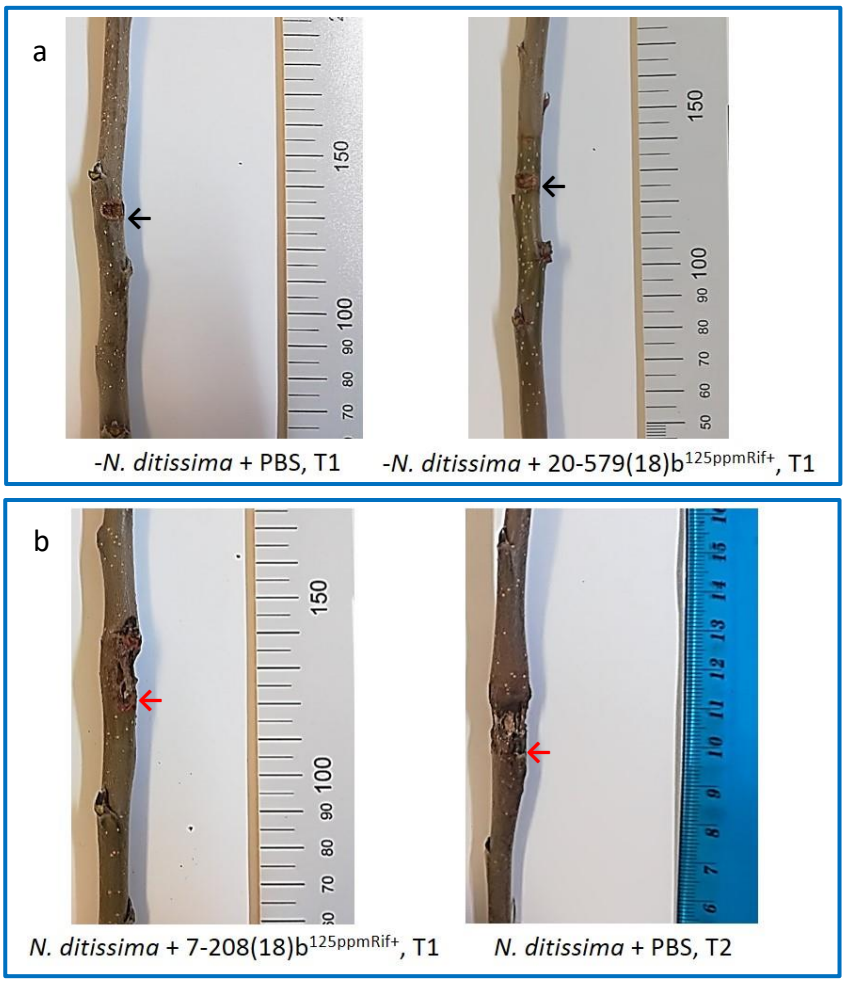

Figure 5.4 Lesions which developed on apple shoots at the inoculation point (the inoculation point is indicated with black arrow) for the different treatments. a) No lesion development for shoots inoculated with Tween 20 (-*N. ditissima*) followed 14 days later with PBS/20-579(18)b$^{125ppmRif+}$ at the 8 weeks assessment (T1); b) lesion development for shoots inoculated with *N. ditissima* followed 14 days later with 7-208(18)b$^{125ppmRif+}$ at the 8 weeks assessment (T1), and shoots inoculated with *N. ditissima* followed 14 days later with PBS at the 16 weeks assessment (T2). Flaky bark, a typical European canker symptom, surrounding the lesion is indicated by the red arrows.

5.4 Discussion

The *Pseudomonas* strains mutated to be rifampicin-resistant were shown to endophytically colonise the detached apple shoot tissue without being pathogenic (Chapter 4). Their ability to suppress *N. ditissima* colonisation *in planta* were investigated in this Chapter. Although the two mutant strains did not reduce infection by *N. ditissima*, there was an indication that inoculation of the apple shoots with these strains prior to *N. ditissima* limited the spread of the pathogen. This suggests that the approach taken in this research has promise to identify strains that may help protect apples against European canker, for which an efficient biocontrol agent has not yet been found (Walter et al., 2017a; Walter et al., 2017b).

No infection was observed with inoculation of *N. ditissima* produced on CMA at 10^5 conidia/mL (10^3 conidia/wound) despite infection occurring with lower concentration 10^4 conidia/mL (10^2 conidia/wound) at the same assessment time. This was probably due to experimental errors as this inoculum concentration resulted in infection at the 28 day assessment, indicating the conidia had germinated and infected the shoots. Failure to reisolate *N. ditissima* from the shoots may also be because the pathogen mainly caused superficial infection 14 days after inoculation, which would be killed by the surface sterilisation process. Crowdy (1949) and Zeller (1926a) reported *N. ditissima* can cause superficial infections by exploiting tissues outside the xylem and then later in the infection process penetrate the xylem to cause infection of parenchyma, vessel and fibre tissues. Surface sterilisation method was reported to affect the isolation frequency of endophytic fungi from leaves of *Neolitsea dealbata* (Lauraceae) (Paulus & Gadek, 2003). The use of qPCR with species specific primers, such as those developed by Ghasemkhani et al. (2016), could be used in conjunction with a milder surface sterilisation protocol to determine the colonisation of the different shoots by the pathogen, including superficially infected tissue. This would also provide information on the effect of inoculation with endophytic bacteria with potential antagonistic activity on different forms of colonisation by *N. ditissima*. Further the use of a pre-treatment with PMA, as used in Chapter 2, could help determine the proportion of live and dead fungal biomass.

The colonisation ability of the three *Pseudomonas* rifampicin-resistant mutant strains in detached shoots and two of them in attached shoots was not affected by the introduction of *N. ditissima*, revealed by the similar pattern in persistence, movement and recovery frequency from the inoculated shoots compared with the control. There was an indication that at the 16 weeks assessment, inoculation with *N. ditissima* may have increased colonisation by the endophytes, regardless of inoculation order of the endophyte and the pathogen. In contrast, Wu (2017) observed that inoculation with the pathogen *Neofusicoccum parvum* reduced colonisation of the

stems of one-year old kiwifruit (*Actinidia chinensis*) plants by the endophytic bacterium *Burkholderia* sp. W4R11 irrespective of whether the pathogen was inoculated 48 h before, simultaneously or after the endophyte inoculation. No reduction in the colonisation ability of endophytes by *N. ditissima* may indicate the endophytes had strong niche occupancy and unable to be displaced by *N. ditissima*. Colonisation is a key factor in selection of endophytes for biocontrol agents as a biocontrol strain has to compete with the indigenous microbial community with or without the presence of the pathogen (Schreiter et al., 2018). Although a biocontrol effect by the endophyte was not observed in the shoot assay in the current study this result showed that the techniques used were useful to observe colonisation as a trait. Another possible reason for enhanced endophyte growth could be that *N. ditissima* may release nutrients (through degradation of plant material) which the endophytic bacteria could utilise to boost their colonisation. Blakeman (1973) found spores of *Botrytis cinerea* released nutrients that supported larger numbers of bacteria. It is also likely that *N. ditissima* and endophytic bacteria occupied different internal tissue niches. This is in contrast to Wu (2017) who showed that the inoculation of both the endophyte and the pathogen reduced the amount of kiwifruit stem segments with recovery of the endophyte and pathogen. Further work using qPCR and/or microscopy could be used to determine the spatial and temporal patterns of colonisation by both the endophytes and the pathogen.

The population size of the test endophytes at the inoculation point of both the detached and attached shoots at the second assessment time were all approximately 4.0 $\log_{10}$ CFU/cm stem section in each treatment. Similar results were reported by Wicaksono et al. (2018) with the population of three chloramphenicol-resistant mutant *Pseudomonas* strains at the inoculation point in stems of kiwifruit (*Actinidia deliciosa*) plantlets being around 4.0 $\log_{10}$ CFU/cm stem segment. With the same method for extracting stem sap for bacterial quantification, the two studies may reflect the maximum number of bacteria that can colonise the plant stem tissue. If this is indeed the carrying capacity of the stem tissue then it is unlikely that inoculation of the wounds with more bacteria would result in higher colonisation for more effective inhibition of the pathogen infection of the wound site. Chen et al. (1995) found that the cotton stems inoculated with a higher inoculation concentration of endophytic bacteria had a higher colonisation population in stem segments immediately after inoculation, while the bacterial population in both of the concentration treatments reached a similar peak after 3 days. However, where these inoculated strains are colonising the internal parts of the plant is not known.

Although all three *Pseudomonas* mutant strains could effectively colonise the detached apple shoots, neither the wildtype or mutant strains showed biocontrol activity by decreasing colonisation by *N. ditissima* in the detached shoot assay. Similar results were reported by Whitaker and Bakker (2018) with endophytic bacterial isolates that showed *in vitro* antagonism towards the wheat fungal pathogen *Fusarium graminearum* unable to reduce the pathogen biomass in detached wheat heads as estimated by qPCR. In contrast, Ren et al. (2013), Wicaksono et al. (2017) and Wicaksono et al. (2018) reported the endophytic bacteria demonstrating biocontrol activity *in vitro* maintained their biocontrol activity in stem tissues. Mercado-Blanco et al. (2004) found that whilst *P. putida* strains showed higher *in vitro* antagonism of the pathogen *Verticillum dahliae* than *P. fluorescens* strains, *P. fluorescens* strains were more effective at suppressing Verticillium wilt i*n planta*. The inconsistent biocontrol efficacy of candidates when tested *in planta* may reflect that, although whilst under optimal laboratory conditions the strains were able to reduce *N. ditissima* colony growth, this is not reflective of their ability to reduce pathogen growth *in vivo* (Whitaker & Bakker, 2018). Their inhibitory activity under high nutrient availability provided by the agar medium in the *in vitro* dual plate assay may not reflect their activity in a nutrient limited environment *in planta* since antibiotic or other inhibitory secondary metabolites may only be produced under nutrient rich conditions, or in the presence of a particular nutrient. Whipps (1987) reported the production of volatile and non-volatile antibiotics by antagonists and the response of the pathogens to these antibiotics were affected by media type. Ownley et al. (2003) showed that differences in the physical and chemical properties of soils affected the biocontrol performance of *Pseudomonas fluorescens* 2-79RN10 against the take-all of wheat pathogen *Gaeuannomyces gramini*s var. *tritici*. In addition, it could also be because of the low biocontrol properties of the three endophytes tested in this study as revealed by the results of the dual culture plating assay in Chapter 3. Another possible reason could be that the endophytic bacteria colonised a different niche within the apple shoot to that of *N. ditissima* during the experimental period. Microscopic analysis by fluorescence *in situ* hybridisation (FISH) and confocal microscopy would be useful to determine where the endophytes are colonising in relation to the pathogen (Compant et al., 2011; Gamalero et al., 2004).

As the three endophytes used in these experiments were not the most effective isolates at inhibiting the *in vitro* growth of *N. ditissima* (Chapter 3), the three *Bacillus* isolates (42-1206(19)b, 21-606(28)b and R3L-6b) shown to be more effective in the dual culture plating assay need to be tested. They were not tested in the current study due to the lack of colonisation of apple shoots by their respective antibiotic mutant strains (Chapter 4). Further work with the wild type strains of the three *Bacillus* isolates is recommended to determine if the mutation related to rifampicin

resistance affected their colonisation ability. Chauhan and Nautiyal (2010) found that the colonisation ability of spontaneous rifampicin-resistant mutant of *Pantoea agglomerans* NBRISRM (SRM) was reduced as compared to the wild type, due to the disruption of *purB* gene which is required for rhizosphere colonisation in *P. agglomerans*. In addition, the other selected bacterial endophytes for which mutants were not successfully produced (Chapter 4) should also be investigated for their biocontrol activity against *N. ditissima in planta*. Tracking of the endophytic colonisation of those isolates could be achieved by other methods such as nucleic acid-based FISH and isolate specific qPCR (Rilling et al., 2019).

In the attached shoot assay, inoculation with *Pseudomonas* sp. isolate 20-579(18)b$^{125ppmRif+}$ followed by *N. ditissima* restricted the movement distance of *N. ditissima* at the 8 week assessment, but this was not observed at the 16 week assessment. This was not observed for the shoots inoculated with 7-208(18)b$^{125ppmRif+}$ followed by *N. ditissima* at either assessment times. This indicated that inoculation of 20-579(18)b$^{125ppmRif+}$ inhibited early shoot colonisation by *N. ditissima* when pre-inoculated, but long-term biocontrol was not achieved. However, this result was based on the presence or absence of *N. ditissima* in inoculated shoots, qPCR is required to determine the relative biomass level of *N. ditissima* in shoots from the different treatments. The ability to provide long-term suppression of the pathogen is normally dependent on the capacity of the biocontrol agent to persist at high colonisation levels in the plant tissue (Lang et al., 2018; Vainio et al., 2001; Wicaksono et al., 2018). Thus, the inability of 20-579(18)b$^{125ppmRif+}$ to limit the pathogen colonisation after 8 weeks could be related to its population size and distribution in the apple shoot tissue. Therefore, it would have been useful to assess the endophytes population size in the different stem sections to determine if the levels differ at the two assessment times, and their potential correlation to *N. ditissima* colonisation.

Both endophyte candidates showed potential as wound protectants when they were pre-inoculated in attached apple shoots, as shown by the reduced recovery frequency of *N. ditissima* from the stem sections surrounding the wounded inoculation point. This was seen at both assessment times for shoots inoculated with 20-579(18)b$^{125ppmRif+}$ and at the second assessment time for shoots inoculated with 7-208(18)b$^{125ppmRif+}$. Application of bacterial antagonists to inhibit infection by pathogens that infect woody plants through wounds has been widely investigated. For example, pre-inoculation of a *Bacillus subtilis* strain to fresh pruning wounds on grapevine reduced infection by the dieback pathogen, *Eutypa lata* (Ferreira et al., 1991). Similarly, Wicaksono et al. (2017) reported that pre-inoculation of wounds created on grapevine canes by two strains of *Pseudomonas* spp. reduced the disease severity caused by *Neofusicoccum* spp.

Wound protection could be attributed to the capacity of the endophytes to effectively colonise wounds as discussed previously. However, the nature of the interaction of the inoculated strains with *N. ditissima* is unknown.

Inoculation of the two *Pseudomonas* endophytic bacteria strains 14 days after inoculation with *N. ditissima* enhanced the movement of *N. ditissima* in shoots and the recovery frequency in the tissue surrounding the inoculation points at the 16 week assessment in attached shoots. This indicated that the application timing of the potential biocontrol agents affected its interaction with *N. ditissima* in apple shoots. Similarly, Alexandrova et al. (2001) reported that application of the antagonist (*Bacillus subtilis* strain BS-F3) 24 h after inoculation of the pathogen produced a lower level of protection against fire blight on pear flowers compared to that achieved when the antagonist was inoculated 24 h before the pathogen. Manohar Jebakumar and Selvarajan (2018) observed that pre-inoculation of banana with banana bunchy top virus (BBTV) prior to the antagonist strains, including *Bacillus pumilus*, *B. subtilis* and *Pseudomonas fluorescens,* significantly reduced biocontrol efficacy, resulting in increasing percent infection and shortening the number of days until expression of symptoms, as compared to the treatment where the virus was inoculated after the antagonists. Wu (2017) also showed that the recovery of the test pathogen *Neofusicoccum parvum* was affected by the inoculation order of *Burkholderia* sp. W4R11C, and the pathogen, with simultaneous inoculation being the most effective in reducing the pathogen recovery. The results indicated that these potential biocontrol agents were not efficient in providing curative effects once the pathogen has invaded into the plants.

Shoots inoculated with *Pseudomonas* sp. isolate 7-208(18)b$^{125ppmRif+}$ before *N. ditissima* produced shorter lesion than for the shoots inoculated with *N. ditissima* before the two endophytic *Pseudomonas* sp. isolates at 8 weeks. This could be due to the *N. ditissima* being inoculated 2 weeks earlier in the treatment of *N. ditissima* followed by endophyte, as compared to the treatment of endophyte prior to *N. ditissima*. However, it is interesting that the length of lesions at 16 weeks were similar for the two inoculation timings. It warrants further investigation on whether 7-208(18)b$^{125ppmRif+}$ had some effect at reducing lesion length.

In this study, biocontrol activity was only assessed in detached and attached shoots of 'Royal Gala'. There is a complex interaction between an introduced biocontrol candidate, plant and the target pathogen (Vinale et al., 2008). Ghasemkhani et al. (2016) found that colonisation of different apple cultivars by *N. ditissima* differed. Therefore, potentially the colonisation of the different apple cultivars by endophytes could also differ because of the interaction between pathogen and endophyte colonisation. In addition, as the three test strains were isolated from

stems of 'Braeburn', 'Scifresh' and 'Grimes Golden', respectively, they might colonise other cultivars better and show a different effect on *N. ditissima*. Smith and Goodman (1999) and Leeman et al. (1995) found that disease suppression of a biocontrol agent was affected by the resistance of the cultivar to the target pathogen where the cultivar resistance was influenced by the pathogen dose. It showed inoculation concentration of the pathogen could affect the biocontrol effect of a biocontrol agent. In this study 10^2 conidia/wound of *N. ditissima* was used and this is a much higher concentration than the minimal concentration required for initiating infection in a shoot assay which was 12 conidia/wound (Walter et al., 2016). Using a lower concentration of *N. ditissima* could allow the endophyte strains to exert a biocontrol effect. It may also be worth testing their biocontrol efficacy under field conditions, with the inoculation concentration of *N. ditissima* at 10-30 conidia/wound, the minimal concentration required for infection in the field (Walter et al., 2016). Future work is required using more apple cultivars with a range of reported resistance to European Canker to determine both the ability of the endophyte strains to colonise the apple stem tissues and to provide control of *N. ditissima* infection. Testing the effect on *N. ditissima* conidial germination could be an effective way for evaluating the control effect, as it was used by Kanto et al. (2007) to evaluate suppression effect of silicate treatment in powdery mildew of strawberry. This should also involve the use of different inoculation concentrations of the pathogen and the endophytes.

In conclusion, although the results of the study indicated that inoculation with the endophyte strains limited the establishment and spread of *N. ditissima* in the apple shoots, long term control of *N. ditissima* was not observed. Future work to attempt to increase the level of control achieved is required, this could include focusing on the effect of combined use of different endophyte biocontrol candidates, application interval and application times.

Chapter 6 Concluding discussion

The overall aim of this thesis was to identify endophytes of apple which could inhibit infection by *N. ditissima* and show their potential to be used as biocontrol agents of European canker. In attached apple shoots, both endophyte candidates showed potential to reduce extent of colonisation when pre-inoculated. However, biological control effect was not found. However, this study advanced the understanding of factors influencing endophyte community in apple shoots, the frequency of antagonistic endophytic bacteria and fungi, *in vitro* biocontrol mechanisms employed by the candidate endophytic bacteria and fungi, and generation of spontaneous antibiotic resistant bacterial mutants as a method to monitor endophytic colonisation by the selected bacteria in apple shoots.

Endophytes living naturally inside plants have potential to be used as biocontrol agents. Previous studies have shown that can produce secondary metabolites such as antibiotics, siderophores and volatile compounds, induce plant resistance and compete with plant pathogens for niches and nutrients (Castignetti & Smarrelli, 1986; Fenton et al., 1992; Gao et al., 2010; Karimi et al., 2012; Vu et al., 2006). Introduction of biocontrol strategies for apple production is expected to reduce the use of chemical fungicides in apple orchards, which will reduce chemical residues in apple fruit and decrease the risk of pathogens developing fungicide resistance. However, in the literature, only arbuscular mycorrhizal fungi (*Funneliformis mosseae* and *Rhizophagus irregularis*) are reported to effectively reduce *N. ditissima* incidence in apple trees (3-year old in pots), which is a significant outcome in the United Kingdom biocontrol study of European canker (Berdeni et al., 2018). Additionally, commercial biocontrol products including Superzyme™ (*Bacillus subtilis, Pseudomonas putida, Trichoderma koningii* and *T. harzianum*), Clarity™ (*B. subtilis*), Fulzyme Plus™ (*B. subtilis*), µInoculant PP9A™ (*P. putida*), Serenade™ Optimum (*B. subtilis*) and Vinevax™ (*T. harzianum*) were not found to effectively control European canker in the field by Walter et al. (2017b) in NZ. This is the first study in NZ on biocontrol potential of endophytes in apple for European canker and one of few international research efforts in this area.

This study is one of six globally that describes microbial associations with the foliage of apples and the first to use DGGE to do so (Chapter 2). A recent study has investigated the endophytes in the apple cultivars ‘Golden Delicious’, ‘Honey Crisp’ and ‘Royal Gala’ (Liu et al., 2018). However, previously published studies have not investigated the effect of the factors including tissue type, site, region and season on the complete endophyte community specifically in apple shoots for ‘Royal Gala’ and ‘Braeburn’. As was expected, tissue type, cultivar and site influenced the composition of endophyte communities and this has been shown for many plant species

throughout the world including endophyte community affected by tissue type for *Viscum album*, *Pinus sylvestris* in Germany and Austria (Peršoh, 2013) and *Vitis vinifera* in Italy (Campisano et al., 2017), by cultivar for potato (*Solanum tuberosum*) in the Netherlands (Andreote et al., 2010) and grapevine (*Vitis vinifera*) in China (Jayawardena et al., 2018), and by site for *Arabidopsis thaliana* and poplar (*Populus* × *euramericana*) in Spain (Knief et al., 2010; Martín-García et al., 2011). However, region had no effect and season had only a small effect on endophyte communities. This is in contrast to previous studies where region was a determinant factor influencing endophytic fungi in various tissues of different plant species, such as leaves of *Ageratina altissima* (Christian et al., 2016) and *Metrosideros polymorpha* (Zimmerman & Vitousek, 2012), roots and shoots of *Dysphania ambrosioides* (Parmar et al., 2018). In addition, season was reported as another important factor related to environmental conditions influencing the endophytic community (Shen & Fulthorpe, 2015), bacterial and fungal endophyte colonisation and/or isolation rate in other plant species (Guo et al., 2008; Mocali et al., 2003; Rather et al., 2018).

The relative abundance of endophyte taxa was not determined in this study. International work is currently using Illumina-based next generation sequencing approaches to describe relative abundance of microbial operational taxonomic units (OTUs) in the rhizosphere of apple (Sun et al., 2014) and soil in apple orchards (Wang et al., 2017; Wang et al., 2016) in China. However , this technique has been used for other fruit species, such as grapevine (Zarraonaindia et al., 2015) and citrus (*Citrus* spp.) (Blaustein et al., 2017). The extension of the work in this thesis to incorporate deep sequencing approaches, such as next generation sequencing, may reveal the effect of region and season on the abundance of endophyte taxa. However, the technology is still low resolution for bacterial taxa as the size of the amplicon is insufficient for high resolution identification of species (Samarajeewa et al., 2015). Of particular interest may be the correlation of abundance and diversity in the endomicrobiome with well-established susceptibility or tolerance to *N. distissima*. This may allow more targeted recovery of endophyte taxa with antagonism towards this pathogen. It might also indicate the most prevalent endophyte groups to target in terms of establishment of endophytic communities.

In this study, foliar tissue type (leaf and stem, two ages) was a major factor influencing the endophyte community of apple in Chapter 2. The microbial endophyte community in leaves typically differed from those in stems for the cultivars assessed (‘Royal Gala’ and ‘Braeburn’). This was consistent with previous studies (da Silva et al., 2013; Sadeghi et al., 2019; Win et al., 2018) and is likely due to both ontogenic differences and the mechanisms that recruit microorganisms to these tissues in apples. Further work is required to determine the contribution of atmospheric

(external) and internal endophyte movement that determines the final composition of leaves. Although no differences in the endophyte community was attributed to region and less difference was attributed to season, this is likely to have been partially due to the reduction of tissue types to solely woody stems, the infection court of the pathogen. Future work that samples a greater range of tissues would be required to test this. The age of the tissues (leaves 2 vs. 6 weeks; stems 2 vs. 3 month) had no effect on fungal communities and only a minor effect on bacteria in stems. This indicated that endophyte accumulation was less affected by the time available for migration of microorganisms into leaves or stems and suggests that the type of plant organ has a stronger effect. This may be functionally related as the leaf plays a major role in photosynthesis while stem is an often lignified support structure (Rascio et al., 1991). The different photosynthetic capacity of them could be related to the variation in endophyte community. Photosynthetic rate in leaves was found to be significantly correlated with bacterial and fungal community (Li et al., 2018). It could also be because stems are not deciduous while leaves are newly formed every year. No effect of tissue age could also be because the age difference (approx. 4 weeks) was small. For example, the endophytic fungal community was found to differ between 1- and 3-year old stem sections of *Viscum* and *Pinus*, but that in 2-year old stem was indistinguishable from the younger and older stems (Peršoh, 2013). Further work to understand the functional role of the acquired endophytes in each tissue may shed light on the drivers of microbial community structure.

This study is the first to show that endophyte communities were significantly different between 'Royal Gala' and 'Braeburn' for all tissue types. In addition, the communities in 'Royal Gala' were more diverse than those found in 'Braeburn', suggesting that they were more tightly regulated in 'Braeburn'. This supports several studies in different plant species using a number of techniques such as cotton (*Gossypium hirsutum*) by culture-dependent method (Adams & Kloepper, 2002), rice (*Oryza sativa*) by PCR-DGGE (Hardoim et al., 2011) and citrus (*Citrus sinensis*) by Illumina sequencing (Blaustein et al., 2017) that show host is a major driver of endophyte recruitment. Although this is a new result for cultivars of apple, research into the structure and function of microbial members of the holobiont is very topical in that the underlying mechanisms of genetic determinants of host recruitment being relaxed or stringent were unknown. Identification and selection for genetic determinants in plants that underpin the recruitment of beneficial bacteria may generate new cultivars which maximise the benefits accrued from microbial partnerships. This process is exemplified by the plant sanction theory. Kiers et al. (2003) reported that soybeans penalise rhizobia that fail to fix N_2 inside their root nodules, thus, restriction of nutrients may be an important mechanism in stabilising a wide range of mutualistic symbioses. Future work could focus on the relationship between apple cultivar and endophyte taxa with biocontrol potential,

such as *Bacillus* and *Pseudomonas* spp. identified in Chapter 3, and the contribution of those endophyte taxa to apple cultivar resistance to European canker.

As the apple trees sampled here were grafted plants, the endophyte community is also likely to be affected by rootstock, or scion-rootstock combination which was reported by Liu et al. (2018). As different scion-rootstock combinations differ in resistance to European canker (Berdeni et al., 2018) and apple cultivar differences in leaf spot resistance are related to their respective endophytic fungal community in leaves (Hirakue & Sugiyama, 2018), a hypothesis can be raised that European canker resistance of apple trees may be related to the endophyte community affected by scion cultivar, rootstock and scion-rootstock combination. This is likely to be a very interesting area of future work, especially as research suggests that a large component of the endomicrobiome is recruited from the rhizosphere (Chi et al., 2005; Compant et al., 2010).

Site was a main driver shaping endophyte community in apple woody stems but not region, however region was assessed only for the reduced dataset of woody stems and site variation was strong within each region. This suggested that local effects from soil conditions and microclimates were stronger than the effect of weather and geographical variations. The site effect may stem from soil conditions and microclimates, including unique environmental factors (rainfall, UV radiation) and management practices, but also the local microbial community. The local microbial community may be influenced by microorganisms being imported, along with the apple plants, from the nursery they were sourced from. Effects on plants from soil microbial communities have been noted in other systems such as the *terroir* of wine (Gilbert et al., 2014). These factors could have an impact on soil and root endophytes which are known to partially define the phyllosphere endophytes due to the migration of a subset of root endophytes up to aerial compartments. For example, Zarraonaindia et al. (2015) reported that soil pH and C:N influenced soil structure and root microbiota of grapevine and changes in leaf- and grape-associated microbiota was correlated to soil carbon even at small spatial scales. In addition to the effect of soil conditions, foliar endophyte communities are more likely to be affected by environmental conditions such as rainfall and UV radiation (Whipps et al., 2008). In addition, given the soil is a reservoir for endophytes, the role of site-related soil conditions could be further explored by comparing endophyte communities between IFP-managed orchards and organic orchards.

Chapter 2 did not address the linkage between the endophyte community and biocontrol. Given the variation between sites within a region, future work to recover potential biocontrol agents of apple diseases could focus more strongly on identifying the specific microbial communities associated with disease-escape phenotypes within sites that have high European canker infection

rates. The complete endophyte community within the stems of trees displaying severely symptomatic or completely asymptomatic disease phenotypes could be compared using Illumina sequencing. The comparative approach has been commonly used to identify biocontrol candidates for different plant diseases such as crown gall infecting almonds, tomato and peach (New & Kerr, 1972), chestnut (*Castanea sativa*) blight by hypovirulence (Heiniger & Rigling, 1994) and Pierce's disease (PD) in grapevine (*Vitis vinifera*) (Deyett et al., 2017). For example, Deyett et al. (2017) investigated microbial community structure and composition in the grapevine cane tissue with severe symptomatic, to mildly symptomatic or asymptomatic phenotypes of PD caused by *Xylella fastidiosa*, and found *Pseudomonas fluorescens* was a potential driver of the disease-escape phenotype and a promising biological control agent of PD.

DGGE fingerprinting technique applied in this study successfully revealed the effect of different factors on both bacterial and fungal endophyte community in apple shoots. However, there are some limitations in DGGE as compared to the next generation sequencing tools (metabarcoding and metagenomics). Firstly, DGGE has a limitation in providing information of abundance of endophytes as previously discussed. Further, next generation sequencing provides a higher resolution of microbial community structure at the phylum and genus level by detecting more taxa with greater accuracy compared to DGGE (Wicaksono, 2016; Yu et al., 2015). However, both DGGE and metabarcoding have limited species-level identification due to the short length of the amplicons (Samarajeewa et al., 2015). Metagenomics technique avoids the limitation of amplicon sequencing because it directly accesses the community genomic information, though it is complicated by uncertainties in assigning genes to specific organisms (Shakya et al., 2013). In addition, RNA-seq of the transcriptome is another alternative method, which provides a linkage between community structure and functional genomics of microbiomes (Urich et al., 2008). mRNA-tags specific for enzymes involved in functional mechanisms of a specific microbial taxa can be revealed by this technique. Kröber et al. (2016) applied high-throughput sequencing of whole transcriptome cDNA libraries of *Bacillus amyloliquefaciens* FZB42 cells to reveal the genes featuring the highest transcription rates in biofilms-forming cells vs. planktonic cells which may contribute to the biocontrol properties of the strain. Thus, in future these newer techniques could build on the knowledge platform generated by this thesis to understand the function of the microbiome in apple plants.

In this study, many plants (LU = 6 trees, HBHV = 78 trees, M1 = 174 trees and M2 = 36 trees), including heritage varieties, across different regions, sites, management practices, cultivars, seasons and European canker infection levels were sampled to produce a culture collection of

1004 bacteria and 332 fungi (representing 39 morphotypes). The 1004 bacteria (from the four samplings) and 87 fungi (from the M1 sampling, representing 33 morphotypes) were used for *in vitro* dual culture assays which identified 18 bacteria (1.8%) and 18 fungi (21%) with antagonism to *N. ditissima*. The proportion of antagonistic bacteria identified in this study was lower than that was reported in previous studies such as 33 out of 192 endophytic bacteria (17%) from mānuka (*Leptospermum scoparium*) inhibited *in vitro* growth of *Ilyonectria liriodendra* (Wicaksono et al., 2016). *In vitro* dual culture screening only selected bacteria and fungi with the biocontrol mechanism of antibiosis, competition and/or direct parasitism, showing the limitation in selecting isolates with host-induced resistance mechanisms. The antagonistic bacterial isolates belong to *Bacillus* spp. (n = 9) and *Pseudomonas* spp. (n = 7), and the antagonistic fungal isolates belong to *Chaetomium* (n = 3), *Epicoccum* (n = 5), *Biscogniauxia* (n = 3), *Penicillium* (n = 1) and a member of the class Dothideomycetes (n = 1), with one fungal isolate unidentified. This is the first study to show a *Chaetomium* sp. as an endophyte of apple leaf and stem. Although not identified further in this thesis, any isolates pursued as potential biocontrol agents would need further identification at the species level, as many genera can include both pathogenic strains and beneficial strains. For example, *Penicillium expansum* and *Biscogniauxia marginata* are apple pathogens (Amiri et al., 2008; Horst, 2013), but *Penicillium* spp. and *B. kuntze* are reported as endophytes (Johnston et al., 2012; Muresan, 2017).

When the cell free filtrate was assessed for the *Bacillus* and *Pseudomonas* isolates a maximum of 30% inhibition was achieved, which contrasted to their strong effect when whole cells were used. Use of live biocontrol agents is inherently variable and in many cases it is also difficult to mass produce the diffusible antimicrobial compounds from the liquid culture. For this reason, medium optimisation is often done to improve propagule yield and/or biocontrol effect by increasing the production of inhibitory compounds based on their reaction to nutrition (Hammami et al., 2009; Jackson, 1997). The inhibitory potential of the filtrate could also be improved by collecting the filtrate when the antibiotic accumulation reaches a peak, which is varied among isolates (Chaiharn et al., 2009; Romero et al., 2007). In addition, some isolates may need the pathogen presence to optimise their inhibitory compounds production ability. For example, the production of the antifungal compound T39butenolide by *Trichoderma harzianum* strain was significantly enhanced by co-cultivation with the fungal pathogens *Rhizoctonia solani* and *Botrytis cinerea* (Vinale et al., 2009). However, it is still complicated by the different inhibitory compounds produced by different isolates, as it was found by Karimi et al. (2012). More consistent results can be achieved when the active ingredient is formulated and used. For example, the application of hydrolytic enzymes, such as chitinases, showed more efficacious biocontrol of fungal pathogens

compared to the use of live bacteria (Neeraja et al., 2010). These are all options to improve the effect from biocontrol agents and should be further investigated if a good candidate is found.

Siderophore production using the modified CAS method (Milagres et al., 1999), showed that *Pseudomonas* spp. produced more siderophores than *Bacillus* spp., yet *Bacillus* spp. were more inhibitory towards *N. ditissima* in the dual culture assay. This indicated that other compounds, such as antibiotics, produced by *Bacillus* spp. were responsible for that antagonism. A similar study by Karimi et al. (2012) found that different combinations of antimicrobial metabolites including proteases, cyanide hydrogen (HCN), siderophores, indole acetic acid (IAA) and cellulases were produced by six *Bacillus* isolates and six *Pseudomonas* isolates as the mode of action for controlling Fusarium wilt of chickpea caused by *Fusarium oxysporum* f. sp. *ciceris*. In their study, the ability to produce siderophores and HCN was variable between *Pseudomonas* isolates (*P. putida* and *P. aueroginosa*), but neither compound was produced by *Bacillus* isolates (*B. subtilis*). Therefore, a combination of *Bacillus* and *Pseudomonas* isolates for inoculation of apple shoots for biocontrol would be more effective as the larger range of antifungal compounds would potentially target the pathogen in multiple ways. For example, combining live cells of two biocontrol agents, *Pichia guilermondii* (yeast) and *Bacillus mycoides*, resulted in additive activity in inhibiting *Botrytis cinerea* conidial germination compared with their separate application (Guetsky et al., 2002).

Two inhibition types were found for endophytic fungi against *N. ditissima* ICMP 14417 in Chapter 3. They were overgrowth of *Chaetomium*, *Biscogniauxia* and *Penicillium* on *N. ditissima* ICMP 14417 (type C), and inhibition zone caused by *Epicoccum* and isolate 3-73f (type D). Biocontrol mechanisms of endophytic fungi include competition, parasitism, production of secondary metabolites, induction of host defences and/or stimulation of plant growth (Porras-Alfaro & Bayman, 2011). As described by Lahlali and Hijri (2010), the two mechanisms of antagonism seen *in vitro* were i) type C , being mainly mycoparasitism or competition for nutrients and space, and ii) type D, being mainly production of antifungal metabolites such as siderophores. However, these traits were observed *in vitro* and may not be the mechanism of action *in vivo*. The latter parts of this thesis focussed on exploring the *in vivo* activity of selected endophytic bacteria, thus further work to investigate the mode of action of the selected endophytic fungi should be done.

In vitro dual culture screening has a bias towards identifying isolates producing antifungal compounds and different screening procedures could result in different isolates being selected. *In planta* assays test the host-antagonist-pathogen interaction and could identify those isolates that have other biocontrol mechanisms, such as induction of plant resistance or competition for

niches/nutrients within plants, which cannot be achieved by *in vitro* dual-culture assays (Pliego et al., 2011). Hanada et al. (2010) screened fungal endophytes for activity against *Phytophthora palmivora* in cacao (*Theobroma cacao*) pods only after they were shown to have colonisation and biocontrol activity *in planta*. Similarly, Evans et al. (2003) screened endophytic fungal isolates from *Theobroma gileri* for their colonisation ability in healthy cocoa (*Theobroma cacao*) tissues first, and then tested those with the ability to colonise the plant for mycoparasitic activity against the fungal pathogen *Crinipellis roreri*, which causes frosty pod rot. However, for many studies, including that presented here, the large number of bacterial and fungal isolates are more efficiently screened using *in vitro* dual-culture assays. This is especially given the seasonal growth of apple and the long latency period (2-3 months) of the pathogen in attached apple shoots.

The development and use of spontaneous antibiotic resistant mutants as a tracking method for colonisation ability of the selected bacteria in apple shoots was successful in this work. However, common limitations, such as inability to force mutants of some bacterial species for some antibiotics and loss of resistance following subculture were experienced. In addition, although comparison between genotype and activity of wildtype and mutant strains was done it is possible that mutations other than those that modify sensitivity to antibiotics were present in the mutant strains. For example, mutation may alter the fitness of the strains *in vitro* as it was found for chloramphenicol resistant mutants in this study (Chapter 4). Mutant strains may also lose or reduce their antibiotic resistance in the natural environment. Compeau et al. (1988) found that *Pseudomonas fluorescens* and *P. putida* rifampicin resistant mutant strains reduced rifampicin resistance when they were recovered from the inoculated soil. Despite of these limitations, the advantages of ease/speed of production and the low regulatory restrictions on these mutants made them the best choice for the glasshouse assays. In future, with access to containment glasshouses, experiments using mutants transformed with *gfp*, *gus*, *hyg B*, or using FISH are alternative ways for tracking colonisation ability of endophytic bacteria (Compant et al., 2011; Germaine et al., 2004; Punja & Utkhede, 2003). Also for strains showing superior biocontrol ability, the development of sequence-characterised amplified regions (SCAR), such as random amplified polymorphic DNA (RAPD), as strain-specific markers could be a useful way to monitor the biocontrol strain (Hermosa et al., 2001; Punja & Utkhede, 2003). Parallel inoculations using the wildtype also allowed the direct comparison of effects between the mutant and wildtype on the host plant to be observed in this study.

Only *Pseudomonas* strains showed colonisation ability in detached shoots of 'Royal Gala' (Chapter 4). This was surprising as all strains were recovered as endophytes from different apple varieties

(three *Pseudomonas* strains isolated from stems of 'Braeburn', 'Scifresh' and 'Grimes Golden', respectively, and three *Bacillus* strains isolated from leaves of 'Royal Gala' and 'Braeburn'). It may indicate the isolates only can colonise the tissue which they were originated, irrespective of variety origin. Overall, colonisation ability of a microbe is plant host dependent which has been investigated by previous studies (Hardoim et al., 2008; Rosenblueth & Martínez-Romero, 2006). Only competent endophytes which have endophytic lifestyles in plant tissues have the potential to be used as biocontrol agents in this way. The endophyte community within leaf and stem of apple was found to be significantly affected by cultivar (also can be called variety) in Chapter 2. However, the difference in the indigenous endophyte community in different varieties did not affect colonisation ability of the three *Pseudomonas* strains. Tissue type was another determinant factor in shaping the indigenous endophyte community in apple shoots (Chapter 2). Therefore, the colonisation ability of *Pseudomonas* and *Bacillus* strains tested in this study may be dependent on the indigenous endophyte community associated with tissue type. In addition, *Bacillus* Rif^{125ppm} mutants may not have been reisolated from the inoculated shoots because they lost or reduced their resistance to rifampicin *in planta*, showing the limitation in using spontaneous antibiotic mutants as monitoring marker as previously discussed. As the three *Pseudomonas* Rif^{125ppm} mutants showed persistence and movement in detached apple shoots, they were further tested for biocontrol activity in detached shoot assay.

Biocontrol potential was found for the two *Pseudomonas* strains 20-579(18)b$^{125ppmRif+}$ and 7-208(18)b$^{125ppmRif+}$ by providing wound protection against *N. ditissima* in the attached shoot assay (Chapter 5). This effect was not observed in the detached shoot assay. This may suggest they required a longer time to show biocontrol effect (approx. 2 months), or that they require a live host to express their phenotype. However, the interaction of the two bacterial mutants with *N. ditissima* within apple stems was unknown. Biocontrol mechanism of endophytic bacteria in controlling fungal pathogens *in planta* are antifungal compound production, competition, induction of systemic resistance in plant and parasitism (Card et al., 2016; Eljounaidi et al., 2016). A combination of several mechanisms can be involved in a biocontrol bacterial endophyte (Chung et al., 2015; Ongena et al., 2007). The mechanism of production of diffusible antifungal compounds was revealed by the *in vitro* biocontrol assay in Chapter 3, such as siderophores. Whether they have the mechanism of inducing systemic resistance in plants could be further tested in future by detecting defense-related genes, such as *PR1* and *PDF1.2* (Niu et al., 2011), in apple shoots when co-inoculated with the potential biocontrol bacteria and *N. ditissima* and compared to those inoculated with *N. ditissima* only. Additionally, it is important to know the colonisation niche of the tested bacterial endophytes and *N. ditissima*, as it may reveal whether

the bacteria reduce colonisation of *N. ditissima* through competition for a niche (Blumenstein et al., 2015).

In vivo biocontrol activity of the selected bacteria being assessed in this study was based on whether they reduced the colonisation ability of *N. ditissima*, but not based on their suppression of European canker symptom. This is because not all infected shoots can show European canker symptoms. In this study, European canker symptoms were not found on the detached shoots infected by *N. ditissima* within the 28 days assessment period, and only found on some attached apple shoots infected by *N. ditissima* at 8-week and 16-week assessments. Similarly, Amponsah et al. (2017a) reported that ≥ 50% asymptomatic wounds on potted apple trees placed onto ASAWA for pathogen isolation yielded *N. ditissima*. There was a limitation in this study that the colonisation assessment of *N. ditissima* in the inoculated shoots were based on presence or absence of the pathogen. The pathogen biomass affected by inoculation of the different test bacteria was not known. For this reason, a quantitative assessment technique is recommended to be used in the future work such as qPCR (Ghasemkhani et al., 2016).

As the two test bacteria did not show obvious biocontrol activity against *N. ditissima* in both shoot assays, either reselection of another strain or enhancement of biocontrol efficacy is required. For the current strains, work should be done on attached apple shoots by inoculating them prior to *N. ditissima* as biocontrol potential was found under this condition in this study. Also, attached apple shoots are more reflective of field conditions than detached shoots in liquid fertiliser. More frequent application of both of the bacterial candidates could be evaluated to improving biocontrol efficacy in the future work. Xu et al. (2019) reported that more applications of a potential biocontrol agent *Bacillus* sp. 7PJ-16 obtained from mulberry stem resulted in lower disease incidence of mulberry fruit sclerotiniose in the field. Alexandrova et al. (2001) also reported that repeated application of *Bacillus subtilis* BS-F3 increased the biocontrol level against fire blight on pear (*Pyrus communis*) flowers and shoots caused by *Erwinia amylovora*, no matter whether it was inoculated before or after the pathogen *E. amylovora*. Further, inoculation intervals between the test biocontrol candidates and the target pathogen could be another factor influencing the biocontrol efficacy. Wu (2017) and Alexandrova et al. (2001) compared 24 h or 48 h inoculation intervals and found this affected the biocontrol efficacy of the candidates *in planta*. Therefore, a shorter inoculation interval is recommended to be assessed in future work. In addition, the combined use of two bacteria as inoculum was not recommended before each of them were confirmed to have *in vivo* biocontrol activity against *N. ditissima*, but dual inoculation could be done in future work.

In conclusion, with the use of *in vitro* biocontrol activity assays, biocontrol candidates of *N. ditissima* were identified from the endophytic bacteria and fungi isolated from apple shoots. *In vivo* biocontrol assay showed the biocontrol potential of two *Pseudomonas* isolates by providing wound protection. It is an important trait for controlling European canker which is a wound disease. The resident endophyte community which could affect colonisation of the introduced endophyte for biocontrol assay was investigated by PCR-DGGE with focus on the effect of tissue type, cultivar, site, region and season. It provided an important knowledge that tissue type, cultivar and site were three determinant factors influencing the apple endophyte community. Challenges related to the development and the use of bacterial endophytes as biocontrol agents are always present, due to the inherent characteristics of the endophyte itself or the environmental conditions. The remaining endophytic bacteria and the selected fungi which were not tested in the *in vivo* shoots assay are a reservoir for future work, which could extend some of the recommendations and ideas developed from the current study.

Conference presentations from this thesis

Liu, J., Ridgway, H. J., Goulard, A., Lin, Y. H., & Jones, E. E. (2016). Biocontrol activity detected in the bacterial endophytes isolated from apple tissues. Poster presented at New Zealand Microbiological Society and NZ Society for Biochemistry and Molecular Biology Joint Annual Conference, Christchurch, New Zealand.

Liu, J., Ridgway, H. J., & Jones, E. E. (2017). Identification of culturable endophytes isolated from apple tissues with antagonism towards *Neonectria ditissima*. Oral presentation at Science Protecting Plant Health Conference, Brisbane, Australia.

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Appendix for Chapter 2

A2.1 Apple tissue samples (highlighted) for analysis of complete microbial endophyte community composition affected by orchard factors including tissue type, region, site, cultivar and season. Apple tissue samples (highlighted and unhighlighted) for analysis of the effects of orchards factors including region, site, cultivar, management practice, season and infection level on the culturable fungal diversity in Chapter 3.

Region	Site	Management practice	Cultivar	Season	Infection level	Block code
Nelson	Site 1	IFP	'Royal Gala'	Spring/Autumn	Low	M1-S1RG(Nel)/ M2-S1RG(Nel)
Nelson	Site 1	IFP	'Braeburn'	Spring/Autumn	Low	M1-S1BN(Nel)/ M2-S1BN(Nel)
Nelson	Site 2	IFP	'Royal Gala' ('Brookfield-redder')	Spring/Autumn	Low	M1-S2RG(Nel)/ M2-S2RG(Nel)
Nelson	Site 2	IFP	'Braeburn'	Spring/Autumn	Low	M1-S2BN(Nel)/ M2-S2BN(Nel)
Nelson	Site 2	IFP	'Scifresh'	Spring	Low	M1-S2SF(Nel)
Nelson	Site 3	IFP	'Royal Gala'	Spring	Low	M1-S3RG(Nel)
Nelson	Site 3	IFP	'Braeburn' ('Mariri Red')	Spring	Low	M1-S3BN(Nel)
Nelson	Site 4	IFP	'Royal Gala'	Spring/Autumn	Low	M1-S4RG(Nel)/ M2-S4RG(Nel)
Nelson	Site 4	IFP	'Braeburn'	Spring/Autumn	Low	M1-S4BN(Nel)/ M2-S4BN(Nel)
Hawke's Bay	Site 5	IFP	'Royal Gala' ('Galaxy')	Spring	Low	M1-S5RG(HB)
Hawke's Bay	Site 5	IFP	'Braeburn'	Spring	Low	M1-S5BN(HB)
Hawke's Bay	Site 5	IFP	'Scifresh'	Spring	Low	M1-S5SF(HB)
Hawke's Bay	Site 6	IFP	'Royal Gala'	Spring	Low	M1-S6RG(HB)
Hawke's Bay	Site 6	IFP	'Braeburn'	Spring	Low	M1-S6BN(HB)
Hawke's Bay	Site 6	IFP	'Scifresh'	Spring	Low	M1-S6SF(HB)
Hawke's Bay	Site 7	Organic	'Royal Gala'	Spring	Low	M1-S7RG-Org(HB)
Hawke's Bay	Site 7	Organic	'Braeburn'	Spring	Low	M1-S7BN-Org(HB)
Hawke's Bay	Site 8	Organic	'Royal Gala'	Spring	Low	M1-S8RG-Org(HB)
Hawke's Bay	Site 8	Organic	'Braeburn'	Spring	Low	M1-S8BN-Org(HB)
Nelson	Site 9	IFP	'Braeburn'	Spring	Low	M1-S9BN(Nel)
Nelson	Site 9	IFP	'Braeburn'	Spring	High	M1-S9BN-Hi(Nel)
Nelson	Site 2	IFP	'Royal Gala'	Spring	High	M1-S2RG-Hi(Nel)
Hawke's Bay	Site 5	IFP	'Scifresh'	Spring	High	M1-S5SF-Hi(HB)

IFP = integrated fruit production programme. In the block code, M1 = spring sampling, M2 = autumn sampling, S = site, RG = 'Royal Gala', BN = 'Braeburn', SF = 'Scifresh', Nel = Nelson, HB = Hawke's Bay, Org = organic orchard, Hi = high infection level of European canker.

A2.2 Recipes of reagents used for DGGE

A2.2.1 2 x DGGE gel loading dye

Component	per 10 mL	Final concentration
2% bromophenol blue	0.25 mL	0.05%
2% xylene cyanol	0.25 mL	0.05%
100% glycerol	7.0 mL	70%
Millipore water	2.5 mL	

A2.2.2 0% and 100% denaturing polyacrylamide (PA) 8% used for total bacteria, α-proteobacteria, β-proteobacteria and total fungi, and 7% used for γ-proteobacteria

0% denaturing PA*	**8% (per 100 mL)**	**7% (per 100 mL)**
40% Acrylamide:Bisacrylamide (37.5 : 1) (Bio-Rad, USA)	20 mL	17.5 mL
50x TAE	1 mL	1 mL
100% glycerol	2 mL	2 mL
Millipore water	to 100 mL	to 100 mL

100% denaturing PA*	**8% (per 100 mL)**	**7% (per 100 mL)**
40% Acrylamide:Bisacrylamide (37.5 : 1) (Bio-Rad, USA)	20 mL	17.5 mL
Urea (Sigma-Aldrich, USA)	42 g	42 g
Formamide (Sigma-Aldrich, USA)	40 mL	40 mL
50x TAE	1 mL	1 mL
100% glycerol	2 mL	2 mL
Millipore water	to 100 mL	to 100 mL

*Store in the dark at room temperature, low heat (≤ 37°C) to dissolve.

A2.2.3 50 x TAE

Component	per litre
Tris Base	242 g
Millipore water	500 mL
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH 8)	100 mL
Millipore water	to 1000 mL

A2.2.4 8 x fixative solution

Component	per litre
96% ethanol	800 mL
Acetic acid	40 mL
Millipore water	160 mL

A2.2.5 1 x fixative solution

Component	per 2 L
8 x fixative solution	250 mL
Millipore water	to 2000 mL

A2.2.6 Silver stain (for 2 gels, to prepare fresh just before staining)

Component	**per 500 mL**
1 x fixative solution	500 mL
Silver nitrate	1 g

A2.2.7 Developer (for 2 gels)

Component	**per ~500 mL**
3% NaOH	250 mL
Millipore water	250 mL
Formaldehyde	1 mL

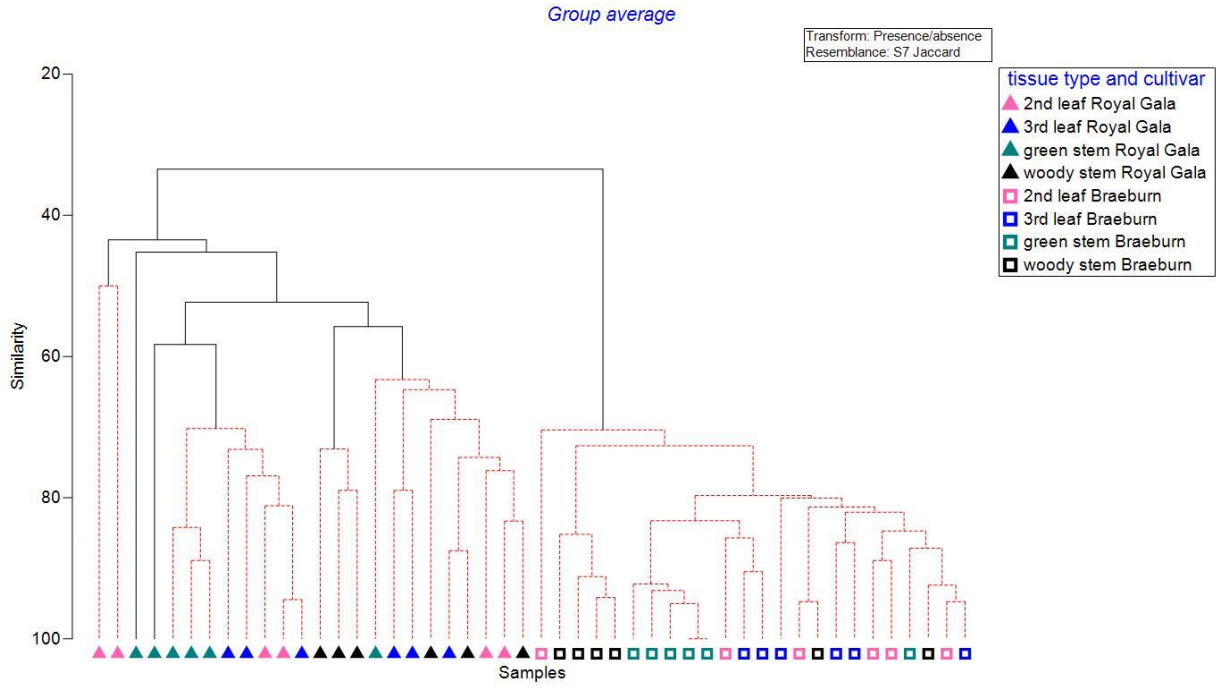
A2.2.8 Cairn's preservation solution

Component	**per litre**
96% ethanol	250 mL
Glycerol	100 mL
Millipore water	650 mL

Total bacteria

Tissue type and cultivar

A2.3 SIMPROF profile using group average cluster analysis of the total bacterial community in two cultivars 'Braeburn' (☐), 'Royal Gala' (▲); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at p = 0.05).



A2.4 Pairwise comparison of total bacterial community similarity and richness in four tissue types of 'Royal Gala' and 'Braeburn' from site 2. Mean of richness of total bacterial community was in the bracket.

Factor	Pairwise comparison of total bacterial community similarity[#]			
Tissue type × cultivar (tissue)	'Royal Gala'	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.262	0.068	0.008*
	3rd leaf		0.045*	0.002**
	Green stem			0.017*
	'Braeburn'	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.073	0.006*	0.018*
	3rd leaf		0.005**	0.002**
	Green stem			0.007*
Tissue type × cultivar (cultivar)	2nd leaf	'*Braeburn*'		
	'*Royal Gala*'	0.005**		
	3rd leaf	'*Braeburn*'		
	'*Royal Gala*'	0.002**		
	Green stem	'*Braeburn*'		
	'*Royal Gala*'	0.007*		
	Woody stem	'*Braeburn*'		
	'*Royal Gala*'	0.006*		
Factor	Pairwise comparison of total bacterial community richness[$]			
Tissue type × cultivar (tissue)	'Royal Gala'	*3rd leaf (16.5)*	*Green stem (18.0)*	*Woody stem (17.2)*
	2nd leaf (14.7)	0.047*	0.001**	0.008*
	3rd leaf (16.5)		0.102	0.461
	Green stem (18.0)			0.357
	'Braeburn'	*3rd leaf (19.8)*	*Green stem (19.0)*	*Woody stem (17.2)*
	2nd leaf (17.8)	0.031*	0.200	0.461
	3rd leaf (19.8)		0.357	0.005**
	Green stem (19.0)			0.047*

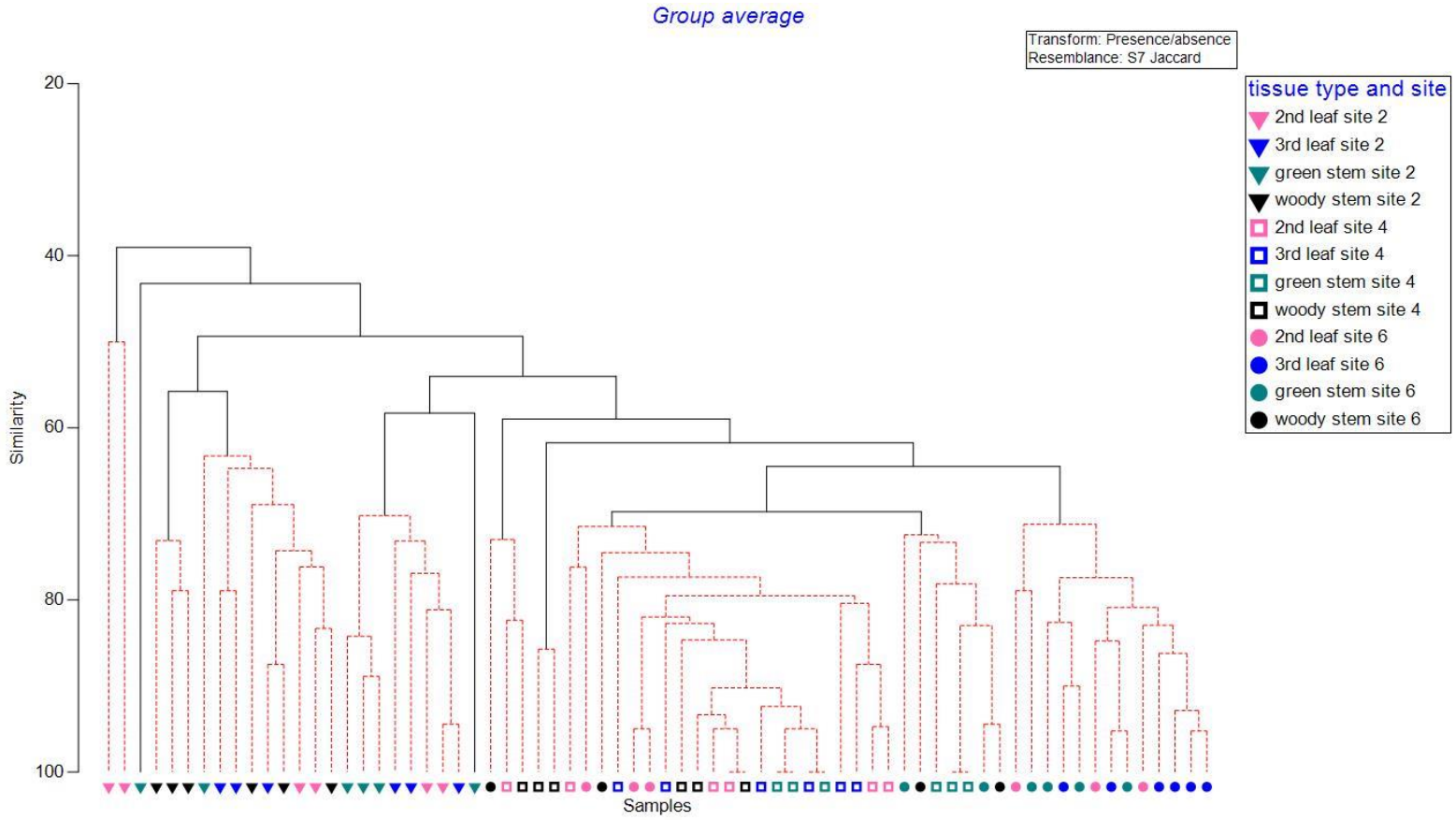
A2.4 Continued

Factor	Pairwise comparison of total bacterial community richness[$]	
Tissue type × cultivar (cultivar)	2nd leaf	'*Braeburn*' *(17.8)*
	'*Royal Gala*' *(14.7)*	0.001**
	3rd leaf	'*Braeburn*' *(19.8)*
	'*Royal Gala*' *(16.5)*	0.001**
	Green stem	'*Braeburn*' *(19.0)*
	'*Royal Gala*' *(18.0)*	0.271
	Woody stem	'*Braeburn*' *(17.2)*
	'*Royal Gala*' *(17.2)*	1.000

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Tissue type and site on 'Royal Gala'

A2.5 SIMPROF profile using group average cluster analysis of the total bacterial community from three sites; site 2 (▼), site 4 (□) and site 6 (●); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at $p = 0.05$).



A2.6 Pairwise comparison of total bacterial community similarity and richness in four tissue types of 'Royal Gala' from site 2, site 4 and site 6. Mean of richness of total bacterial community was in the bracket.

Factor	Pairwise comparison of total bacterial community similarity[#]			
Tissue type × site (tissue)	Site 2	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.297	0.075	0.002**
	3rd leaf		0.048*	0.006*
	Green stem			0.022*
	Site 4	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.191	0.040*	0.176
	3rd leaf		0.112	0.065
	Green stem			0.436
	Site 6	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.023*	0.478	0.066
	3rd leaf		0.333	0.007*
	Green stem			0.221
Tissue type × site (site)	2nd leaf	*Site 4*	*Site 6*	
	Site 2	0.002**	0.003**	
	Site 4		0.288	
	3rd leaf	*Site 4*	*Site 6*	
	Site 2	0.002**	0.001**	
	Site 4		0.001**	
	Green stem	*Site 4*	*Site 6*	
	Site 2	0.002**	0.009*	
	Site 4		0.007*	
	Woody stem	*Site 4*	*Site 6*	
	Site 2	0.002**	0.011*	
	Site 4		0.241	
Factor	Pairwise comparison of total bacterial community richness[$]			
Site		*Site 4 (19.0)*	*Site 6 (19.2)*	
	Site 2 (16.9)	< 0.001**	< 0.001**	
	Site 4 (19.0)		0.658	

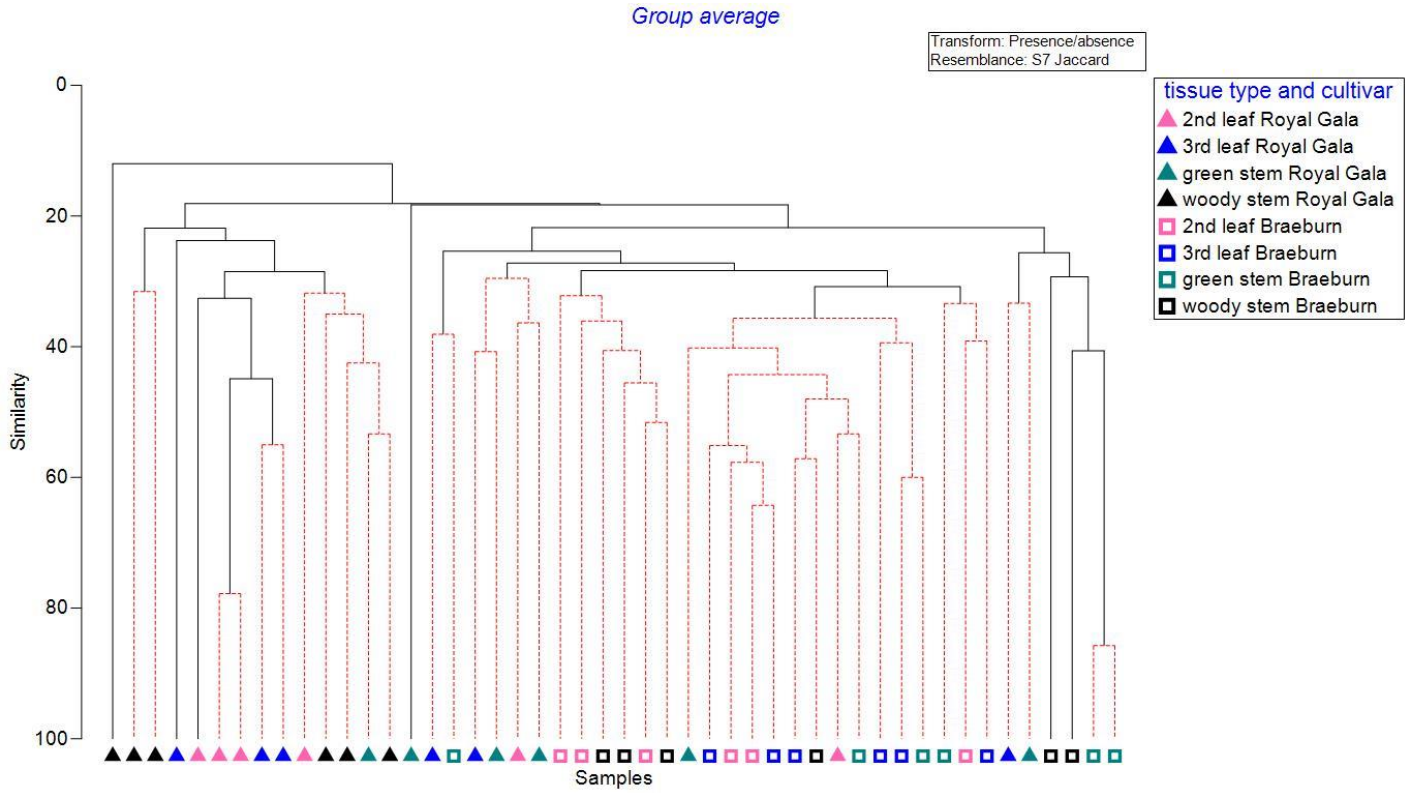
# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Total fungi

Tissue type and cultivar

A2.7 SIMPROF profile using group average cluster analysis of the total fungal community in two cultivars 'Braeburn' (□), 'Royal Gala' (▲);

Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at $p = 0.05$).



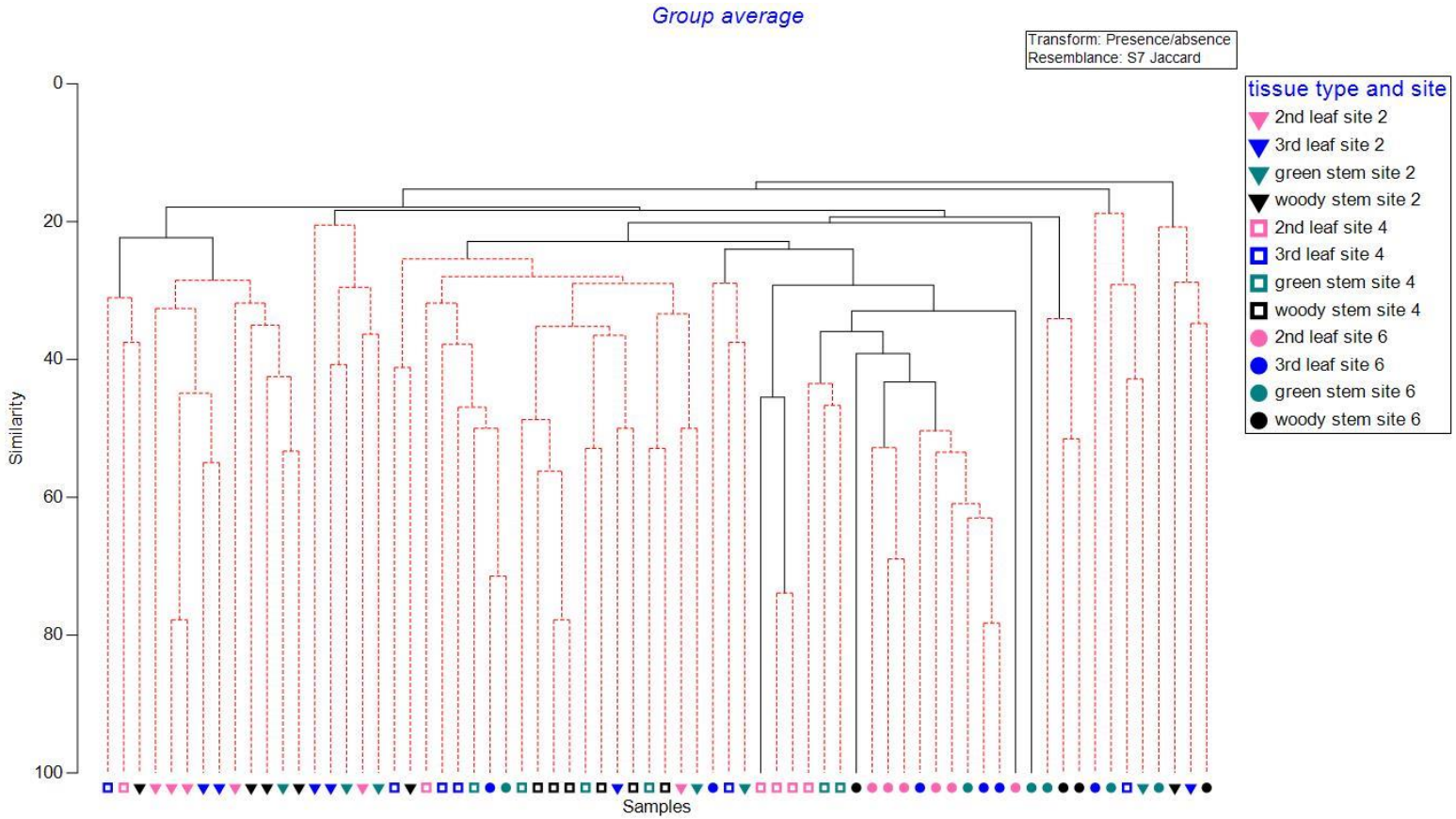
A2.8 Pairwise comparison of total fungal community similarity and richness in four tissue types of 'Royal Gala' and 'Braeburn' from site 2. Mean of richness of total fungal communities was in the bracket.

Factor	Pairwise comparison of total fungal community similarity[#]			
Tissue type × cultivar (tissue)	'Royal Gala'	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.423	0.159	0.045*
	3rd leaf		0.743	0.143
	Green stem			0.073
	'Braeburn'	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.422	0.120	0.053
	3rd leaf		0.388	0.005**
	Green stem			0.091
Tissue type × cultivar (cultivar)	2nd leaf	'*Braeburn*'		
	'*Royal Gala*'	0.002**		
	3rd leaf	'*Braeburn*'		
	'*Royal Gala*'	0.005**		
	Green stem	'*Braeburn*'		
	'*Royal Gala*'	0.082		
	Woody stem	'*Braeburn*'		
	'*Royal Gala*'	0.001**		
Pairwise comparison of total fungal community richness[$]: All factors were not significant.				

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Tissue type and site on 'Royal Gala'

A2.9 SIMPROF profile using group average cluster analysis of the total fungal community from three sites; site 2 (▼), site 4 (□) and site 6 (●); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at $p = 0.05$).



A2.10 Pairwise comparison of total fungal community similarity and richness in four tissue types of 'Royal Gala' from site 2, site 4 and site 6. Mean of richness of total fungal community was in the bracket.

Factor	Pairwise comparison of total fungal community similarity[#]			
Tissue type × site (tissue)	Site 2	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.412	0.179	0.034*
	3rd leaf		0.716	0.167
	Green stem			0.072
	Site 4	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.032*	0.01*	0.004**
	3rd leaf		0.238	0.008*
	Green stem			0.091
	Site 6	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.023*	0.006*	0.005**
	3rd leaf		0.350	0.041*
	Green stem			0.054
Tissue type × site (site)	2nd leaf	*Site 4*	*Site 6*	
	Site 2	0.009*	0.001**	
	Site 4		0.004**	
	3rd leaf	*Site 4*	*Site 6*	
	Site 2	0.007*	0.002**	
	Site 4		0.068	
	Green stem	*Site 4*	*Site 6*	
	Site 2	0.004**	0.004**	
	Site 4		0.001**	
	Woody stem	*Site 4*	*Site 6*	
	Site 2	0.003**	0.002**	
	Site 4		0.004**	

A2.10 Continued

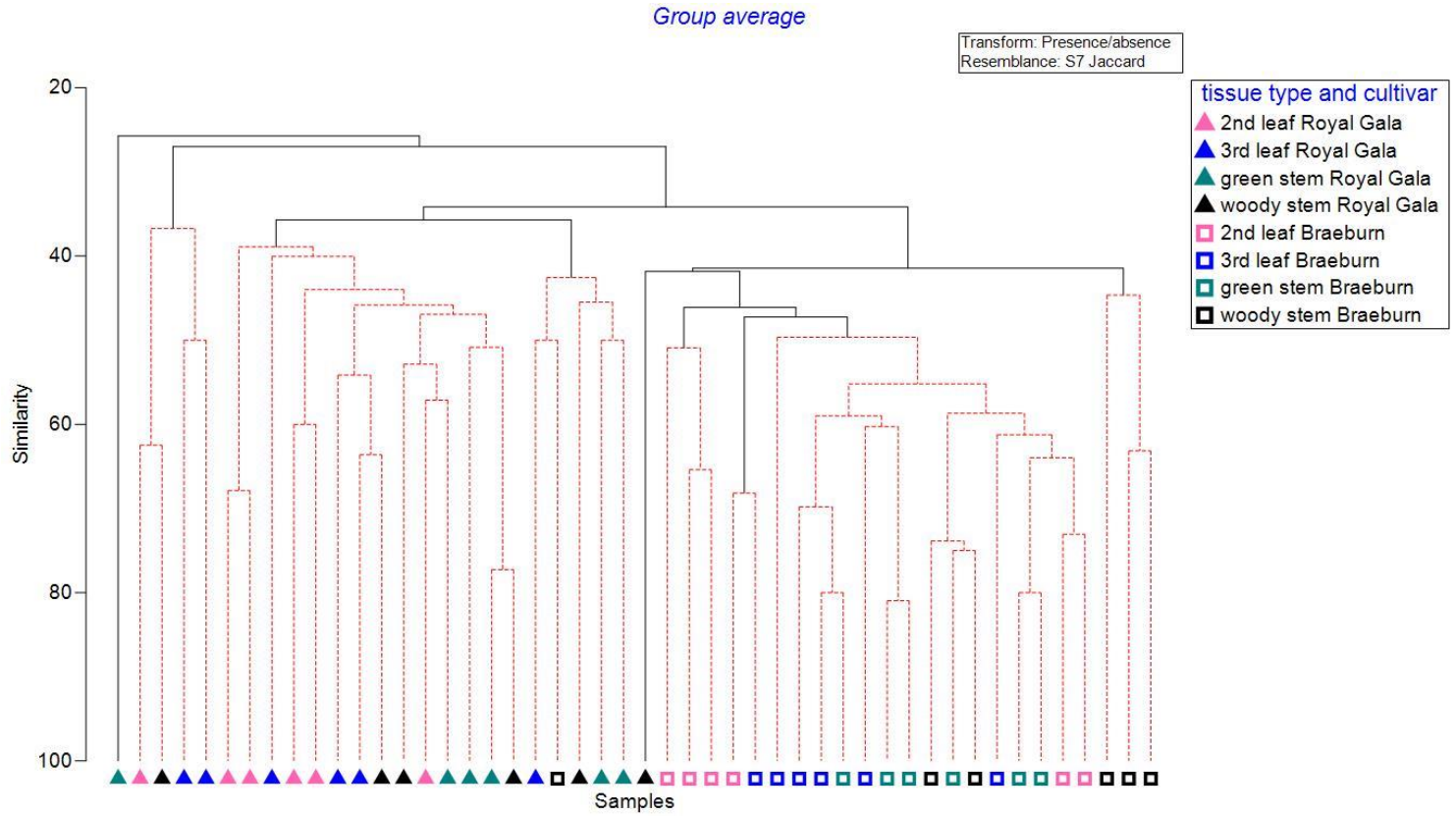
Factor	Pairwise comparison of total fungal community richness[$]			
Tissue type × site (tissue)	Site 2	*3rd leaf (13.7)*	*Green stem (13.5)*	*Woody stem (13.0)*
	2nd leaf (15.2)	0.546	0.503	0.384
	3rd leaf (13.7)		0.946	0.788
	Green stem (13.5)			0.840
	Site 4	*3rd leaf (12.5)*	*Green stem (14.5)*	*Woody stem (9.8)*
	2nd leaf (19.5)	0.006*	0.048*	< 0.001**
	3rd leaf (12.5)		0.422	0.285
	Green stem (14.5)			0.064
	Site 6	*3rd leaf (16.7)*	*Green stem (11.5)*	*Woody stem (22.3)*
	2nd leaf (22.0)	0.035*	< 0.001**	0.928
	3rd leaf (16.7)		0.041*	0.048*
	Green stem (11.5)			< 0.001**
Tissue type × site (site)	2nd leaf	*Site 4 (19.5)*	*Site 6 (22.0)*	
	Site 2 (15.2)	0.085	0.008*	
	Site 4 (19.5)		0.316	
	3rd leaf	*Site 4 (12.5)*	*Site 6 (16.7)*	
	Site 2 (13.7)	0.639	0.230	
	Site 4 (12.5)		0.097	
	Green stem	*Site 4 (14.5)*	*Site 6 (11.5)*	
	Site 2 (13.5)	0.687	0.422	
	Site 4 (14.5)		0.230	
	Woody stem	*Site 4 (9.8)*	*Site 6 (22.3)*	
	Site 2 (13.0)	0.205	0.001**	
	Site 4 (9.8)		< 0.001**	

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

α-proteobacteria

Tissue type and cultivar

A2.11 SIMPROF profile using group average cluster analysis of α-proteobacterial community in two cultivars 'Braeburn' (□), 'Royal Gala' (▲); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at p = 0.05).



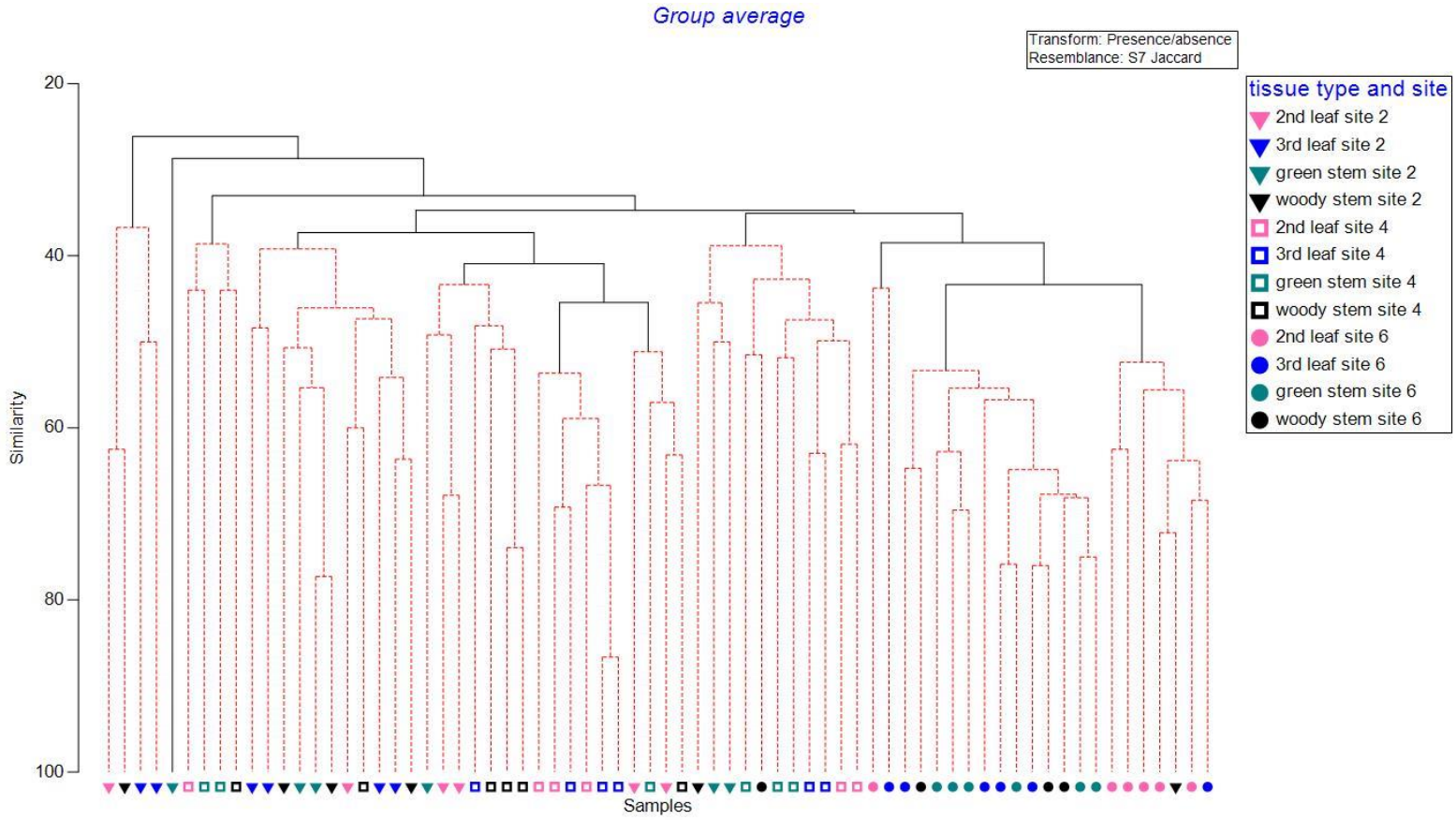
A2.12 Pairwise comparison of α-proteobacterial community similarity and richness in four tissue types of 'Royal Gala' and 'Braeburn' from site 2. Mean of richness of α-proteobacterial community was in the bracket.

Factor	Pairwise comparison of α-proteobacterial community similarity[#]			
Tissue type × cultivar (tissue)	'Royal Gala'	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.269	0.087	0.144
	3rd leaf		0.253	0.087
	Green stem			0.414
	'Braeburn'	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.011*	0.002**	0.002**
	3rd leaf		0.017*	0.002**
	Green stem			0.004**
Tissue type × cultivar (cultivar)	2nd leaf	'*Braeburn*'		
	'*Royal Gala*'	0.002**		
	3rd leaf	'*Braeburn*'		
	'*Royal Gala*'	0.004**		
	Green stem	'*Braeburn*'		
	'*Royal Gala*'	0.001**		
	Woody stem	'*Braeburn*'		
	'*Royal Gala*'	0.002**		
Pairwise comparison of α-proteobacterial community richness[$]: All factors were not significant.				

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Tissue type and site on 'Royal Gala'

A2.13 SIMPROF profile using group average cluster analysis of the α-proteobacterial community from three sites; site 2 (▼), site 4 (□) and site 6 (●); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at p = 0.05).



A2.14 Pairwise comparison of α-proteobacterial community similarity and richness in four tissue types of 'Royal Gala' from site 2, site 4 and site 6. Mean of richness of α-proteobacterial community was in the bracket.

Factor	Pairwise comparison of α-proteobacterial community similarity[#]			
Tissue type × site (tissue)	Site 2	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.297	0.066	0.155
	3rd leaf		0.255	0.101
	Green stem			0.396
	Site 4	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.183	0.104	0.004**
	3rd leaf		0.177	0.309
	Green stem			0.094
	Site 6	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.050*	0.005**	0.006*
	3rd leaf		0.315	0.239
	Green stem			0.052
Tissue type × site (site)	2nd leaf	*Site 4*	*Site 6*	
	Site 2	0.005**	0.002**	
	Site 4		0.004**	
	3rd leaf	*Site 4*	*Site 6*	
	Site 2	0.007*	0.002**	
	Site 4		0.004**	
	Green stem	*Site 4*	*Site 6*	
	Site 2	0.006*	0.001**	
	Site 4		0.001**	
	Woody stem	*Site 4*	*Site 6*	
	Site 2	0.011*	0.004**	
	Site 4		0.005**	
Pairwise comparison of α-proteobacterial community richness[$]: All factors were not significant.				

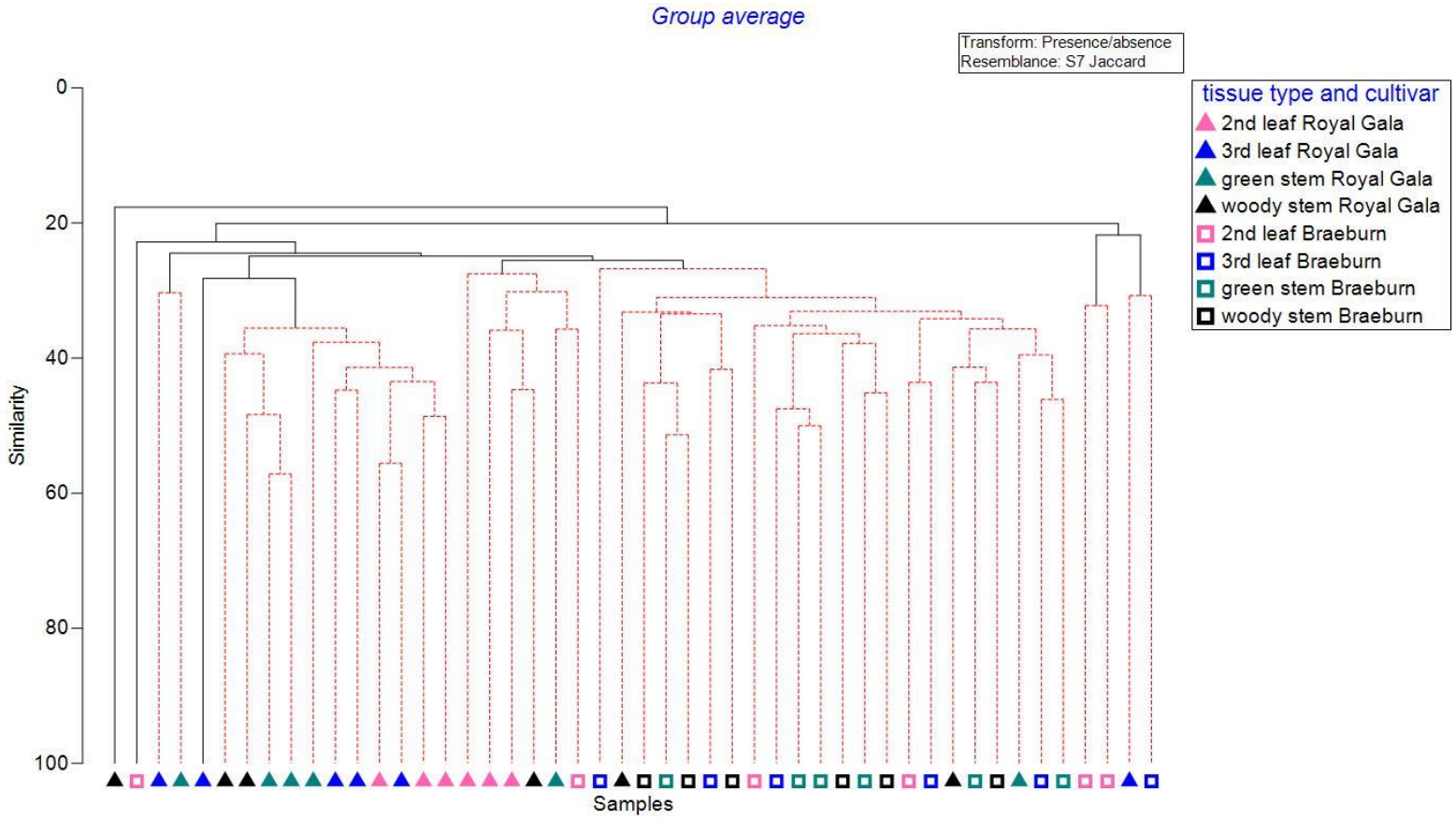
# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

β-proteobacteria

Tissue type and cultivar

A2.15 SIMPROF profile using group average cluster analysis of β-proteobacterial community in two cultivars 'Braeburn' (□), 'Royal Gala' (▲);

Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at $p = 0.05$).



A2.16 Pairwise comparison of β-proteobacterial community similarity and richness in four tissue types of 'Royal Gala' and 'Braeburn' from site 2. Mean of richness of β-proteobacterial community was in the bracket.

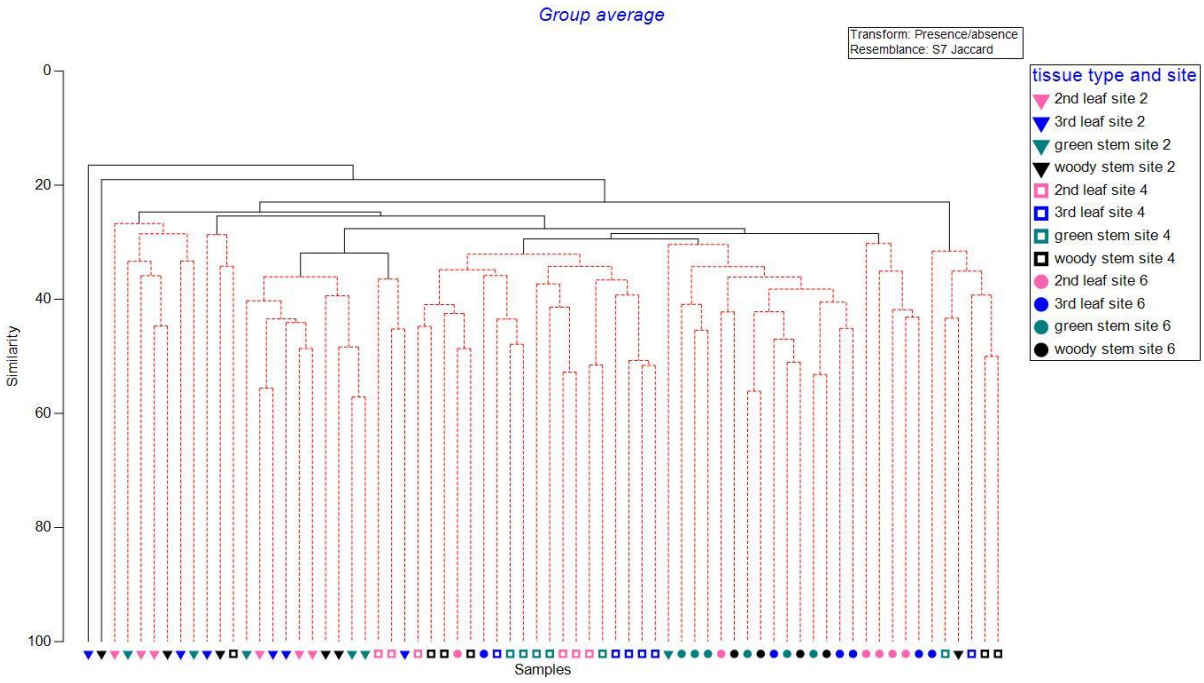
Factor	Pairwise comparison of β-proteobacterial community similarity[#]	
Cultivar		'*Braeburn*'
	'*Royal Gala*'	0.001**
Factor	Pairwise comparison of β-proteobacterial community richness[$]	
Cultivar		'*Braeburn*' *(24.5)*
	'*Royal Gala*' *(28.3)*	0.009*

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA.
$ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Tissue type and site on 'Royal Gala'

A2.17 SIMPROF profile using group average cluster analysis of the β-proteobacterial community from three sites; site 2 (▼), site 4 (□) and site 6 (●);

Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at $p = 0.05$).



A2.18 Pairwise comparison of β-proteobacterial community similarity and richness in four tissue types of 'Royal Gala' from site 2, site 4 and site 6. Mean of richness of β-proteobacterial community was in the bracket.

Factor	Pairwise comparison of β-proteobacterial community similarity[#]			
Tissue type × site (tissue)	Site 2	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.589	0.216	0.239
	3rd leaf		0.566	0.422
	Green stem			0.755
	Site 4	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.013*	0.009*	0.042*
	3rd leaf		0.159	0.208
	Green stem			0.588
	Site 6	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.408	0.004**	0.010*
	3rd leaf		0.420	0.111
	Green stem			0.528
Tissue type × site (site)	2nd leaf	*Site 4*	*Site 6*	
	Site 2	0.003**	0.005**	
	Site 4		0.002**	
	3rd leaf	*Site 4*	*Site 6*	
	Site 2	0.003**	0.007*	
	Site 4		0.003**	
	Green stem	*Site 4*	*Site 6*	
	Site 2	0.003**	0.002**	
	Site 4		0.002**	
	Woody stem	*Site 4*	*Site 6*	
	Site 2	0.065	0.062	
	Site 4		0.016*	

A2.18 Continued

Factor	Pairwise comparison of β-proteobacterial community richness[$]		
site		*Site 4 (25.8)*	*Site 6 (33.3)*
	Site 2 (28.3)	0.087	0.001**
	Site 4 (25.8)		< 0.001**

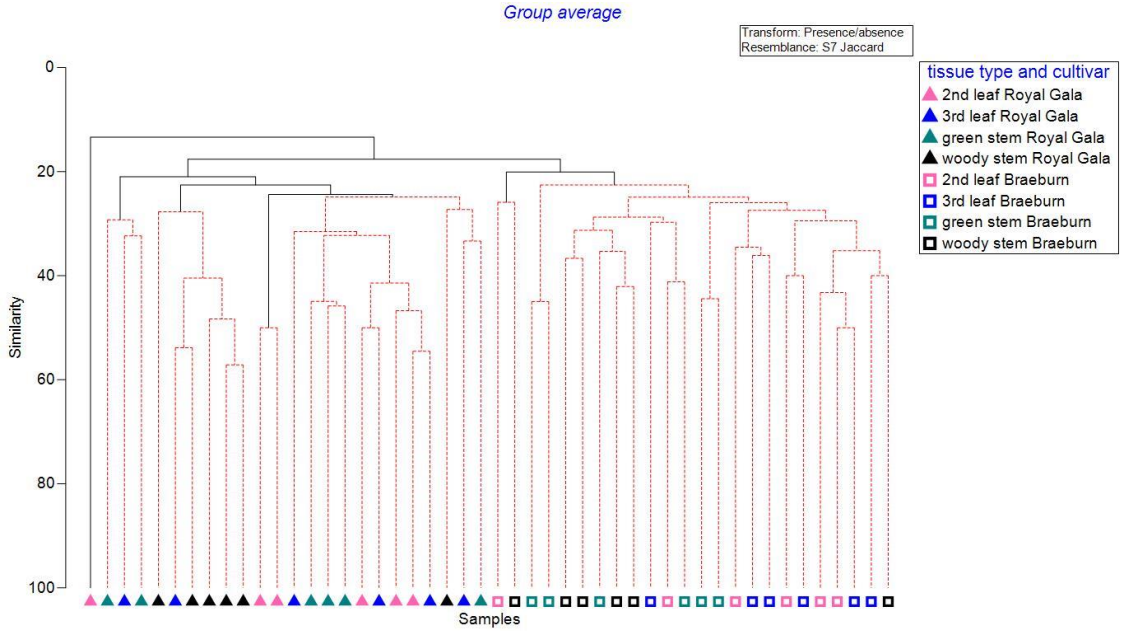
# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

γ-proteobacteria

Tissue type and cultivar

A2.19 SIMPROF profile using group average cluster analysis of γ-proteobacterial community in two cultivars 'Braeburn' (☐), 'Royal Gala' (▲);

Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at $p = 0.05$).



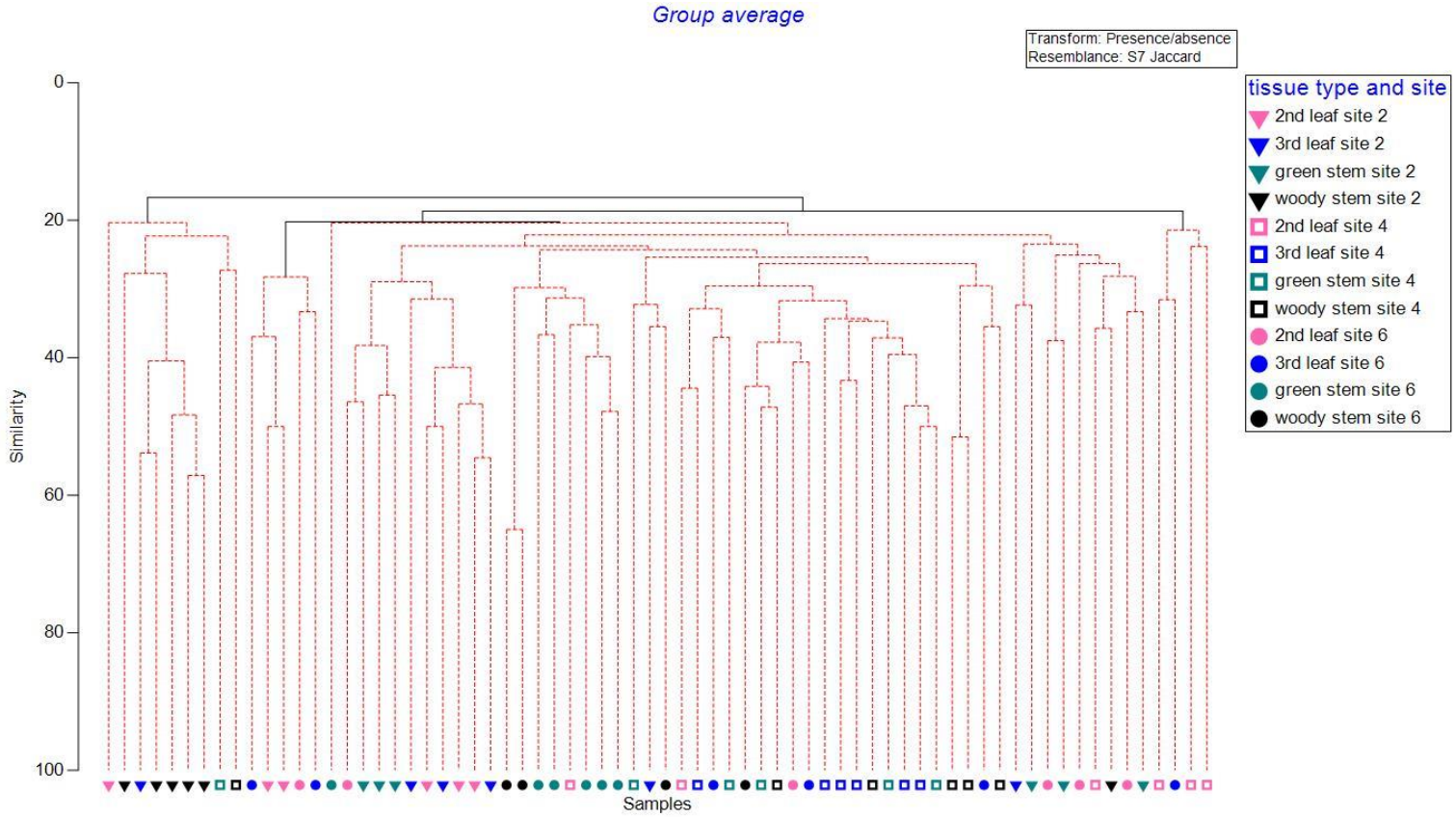
A2.20 Pairwise comparison of γ-proteobacterial community similarity and richness in four tissue types of 'Royal Gala' and 'Braeburn' from site 2. Mean of richness of γ-proteobacterial community was in the bracket.

Factor	Pairwise comparison of γ-proteobacterial community similarity[#]			
Tissue type × cultivar (tissue)	'Royal Gala'	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.532	0.005**	0.016*
	3rd leaf		0.194	0.077
	Green stem			0.004**
	'Braeburn'	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.087	0.013*	0.008*
	3rd leaf		0.108	0.237
	Green stem			0.261
Tissue type × cultivar (cultivar)	2nd leaf	'*Braeburn*'		
	'*Royal Gala*'	0.004**		
	3rd leaf	'*Braeburn*'		
	'*Royal Gala*'	0.002**		
	Green stem	'*Braeburn*'		
	'*Royal Gala*'	0.004**		
	Woody stem	'*Braeburn*'		
	'*Royal Gala*'	0.002**		
Factor	Pairwise comparison of γ-proteobacterial community richness[$]			
Cultivar		'*Braeburn*' *(20.5)*		
	'*Royal Gala*' *(16.8)*	0.012*		

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Tissue type and site on 'Royal Gala'

A2.21 SIMPROF profile using group average cluster analysis of the γ-proteobacterial community from three sites; site 2 (▼), site 4 (□) and site 6 (●); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at p = 0.05).



A2.22 Pairwise comparison of γ-proteobacterial community similarity and richness in four tissue types of 'Royal Gala' from site 2, site 4 and site 6. Mean of richness of γ-proteobacterial community was in the bracket.

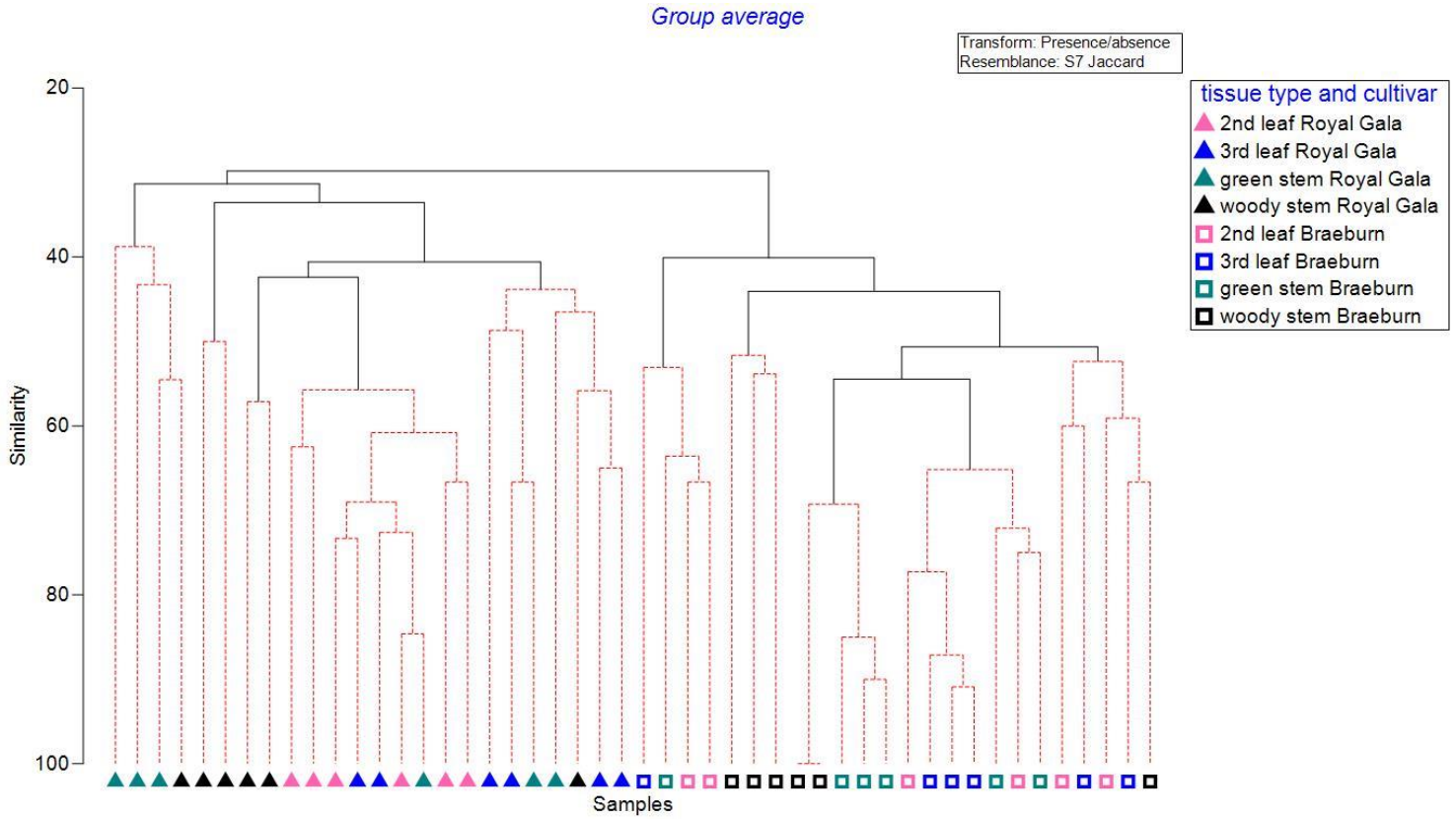
Factor	Pairwise comparison of γ-proteobacterial community similarity[#]			
Tissue type × site (tissue)	Site 2	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.544	0.011*	0.018*
	3rd leaf		0.191	0.063
	Green stem			0.013*
	Site 4	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.090	0.256	0.180
	3rd leaf		0.305	0.004**
	Green stem			0.078
	Site 6	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.114	0.011*	0.488
	3rd leaf		0.003**	0.074
	Green stem			0.169
Tissue type × site (site)	2nd leaf	*Site 4*	*Site 6*	
	Site 2	0.006*	0.008*	
	Site 4		0.108	
	3rd leaf	*Site 4*	*Site 6*	
	Site 2	0.002**	0.004**	
	Site 4		0.004**	
	Green stem	*Site 4*	*Site 6*	
	Site 2	0.010*	0.001**	
	Site 4		0.004**	
	Woody stem	*Site 4*	*Site 6*	
	Site 2	0.004**	0.015*	
	Site 4		0.044*	
Pairwise comparison of γ-proteobacterial community richness[$]: All factors were not significant.				

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Actinobacteria

Tissue type and cultivar

A2.23 SIMPROF profile using group average cluster analysis of actinobacterial community in two cultivars 'Braeburn' (□), 'Royal Gala' (▲); Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at p = 0.05).



A2.24 Pairwise comparison of actinobacterial community similarity and richness in four tissue types of 'Royal Gala' and 'Braeburn' from site 2. Mean of richness of actinobacterial community was in the bracket.

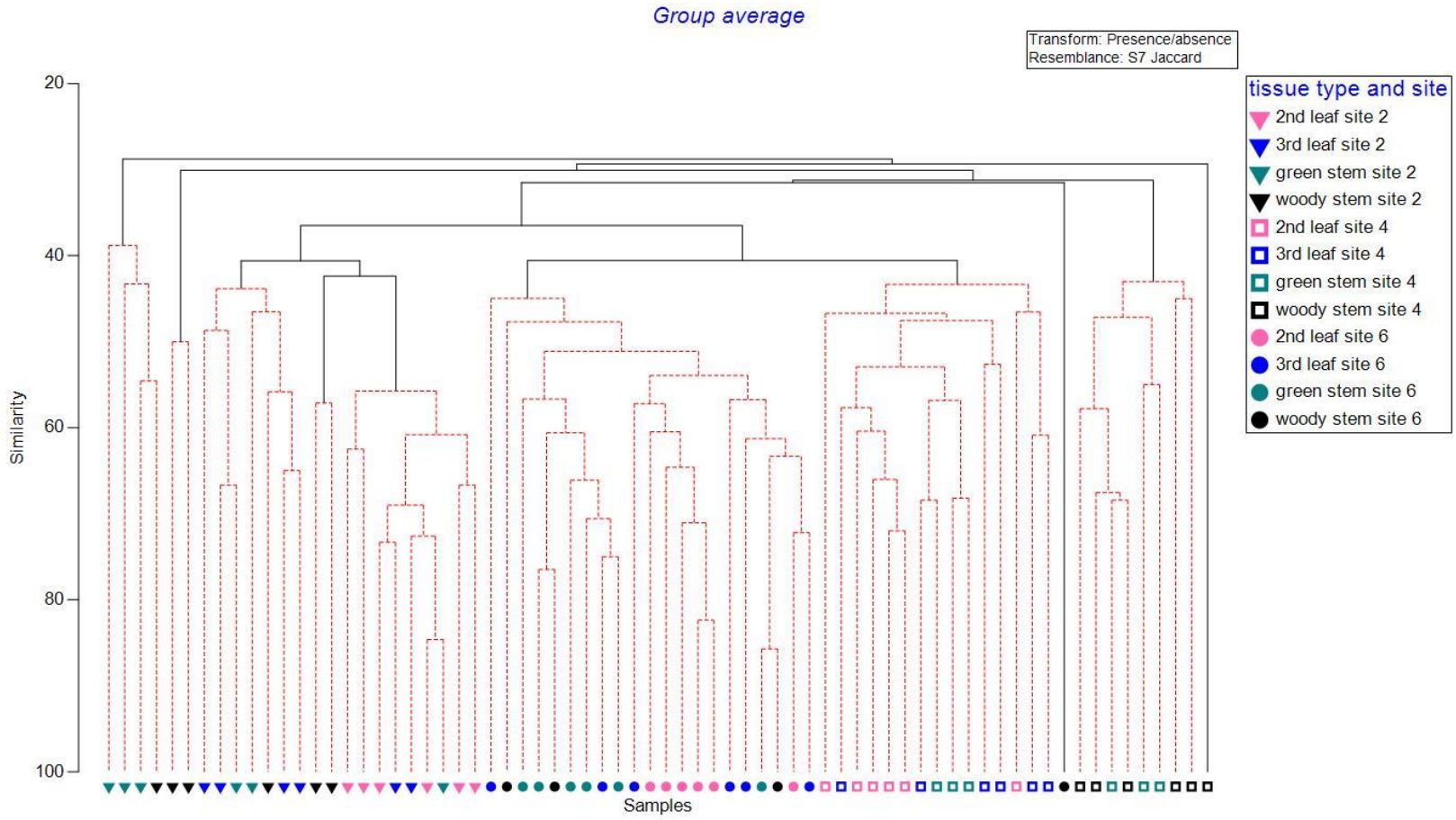
Factor		Pairwise comparison of actinobacterial community similarity[#]		
Tissue type × cultivar (tissue)	'Royal Gala'	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.026*	0.014*	0.004**
	3rd leaf		0.499	0.299
	Green stem			0.192
	'Braeburn'	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.337	0.139	0.004**
	3rd leaf		0.112	0.006*
	Green stem			0.231
Tissue type × cultivar (cultivar)	2nd leaf	'*Braeburn*'		
	'*Royal Gala*'	0.003**		
	3rd leaf	'*Braeburn*'		
	'*Royal Gala*'	0.002**		
	Green stem	'*Braeburn*'		
	'*Royal Gala*'	0.004**		
	Woody stem	'*Braeburn*'		
	'*Royal Gala*'	0.001**		
Factor		Pairwise comparison of actinobacterial community richness[$]		
Tissue		*3rd leaf (13.3)*	*Green stem (11.8)*	*Woody stem (10.8)*
	2nd leaf (12.8)	0.594	0.289	0.038*
	3rd leaf (13.3)		0.115	0.010*
	Green stem (11.8)			0.289
Cultivar		'*Braeburn*' *(10.4)*		
	'*Royal Gala*' *(13.8)*	< 0.001**		

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on general linear model (GLM). * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$)

Tissue type and site on 'Royal Gala'

A2.25 SIMPROF profile using group average cluster analysis of the actinobacterial community from three sites; site 2 (▼), site 4 (□) and site 6 (●);

Second leaf (pink), third leaf (blue), green stem (green) and woody stem (black) (solid line indicates significant difference at p = 0.05).



A2.26 Pairwise comparison of actinobacterial community similarity and richness in four tissue types of 'Royal Gala' from site 2, site 4 and site 6. Mean of richness of actinobacterial community was in the bracket.

Factor	Pairwise comparison of actinobacterial community similarity[#]			
Tissue type × site (tissue)	Site 2	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.015*	0.007*	0.005**
	3rd leaf		0.491	0.330
	Green stem			0.159
	Site 4	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.006*	0.005**	0.011*
	3rd leaf		0.122	0.018*
	Green stem			0.250
	Site 6	*3rd leaf*	*Green stem*	*Woody stem*
	2nd leaf	0.015*	0.002**	0.005**
	3rd leaf		0.017*	0.427
	Green stem			0.523
Tissue type × site (site)	2nd leaf	*Site 4*	*Site 6*	
	Site 2	0.003**	0.003**	
	Site 4		0.003**	
	3rd leaf	*Site 4*	*Site 6*	
	Site 2	0.004**	0.004**	
	Site 4		0.006*	
	Green stem	*Site 4*	*Site 6*	
	Site 2	0.004**	0.001**	
	Site 4		0.005**	
	Woody stem	*Site 4*	*Site 6*	
	Site 2	0.004**	0.022*	
	Site 4		0.025*	

A2.26 Continued

Factor	Pairwise comparison of actinobacterial community richness[$]			
Tissue		*3rd leaf (16.2)*	*Green stem (14.9)*	*Woody stem (13.6)*
	2nd leaf (16.8)	0.462	0.015*	< 0.001**
	3rd leaf (16.2)		0.081	0.001**
	Green stem (14.9)			0.100
Site		*Site 4 (16.9)*	*Site 6 (15.4)*	
	Site 2 (13.8)	< 0.001**	0.027*	
	Site 4 (16.9)		0.023*	

# denotes level of statistical significance of endophyte community similarity based on PERMANOVA. $ denotes level of statistical significance of endophyte community richness based on LSD. * significantly different ($p \leq 0.05$), ** highly significantly different ($p \leq 0.005$).

Appendix for Chapter 3

A3.1 Thirty-five heritage varieties and four commercial varieties collected from Plant & Food Research Orchard in Hawke's Bay (HBHV) for endophyte isolation with/without bark.

Named variety	Plating for isolation	Named variety	Plating for isolation
'Adams Pearmain'	bark	'Winston'	no bark
'Cox's Orange Pippin ELMA 2'	bark	'Grimes Golden'	no bark
'Granny Smith'	bark	'Mr Gladstone'	no bark
'Lady Sudeley'	bark	'Nonpareil'	no bark
'Laxton's Superb'	bark	'Orin'	no bark
'Monty's Surprise'	bark	'Orleans Reinette'	no bark
'Newtown Pippin'	bark	'Northern Spy'	no bark
'Priscilla'	bark	'Red Astrachan'	no bark
'Scarlet Pimpernel'	bark	'Erwin Baur'	no bark
'Sunset'	bark	'Idared'	no bark
'Tydeman's Late Orange'	bark	'Esopus Spitzenberg'	no bark
'Benoni'	bark	'Cornish Aromatic'	no bark
'Early Strawberry'	bark	'Cortland'	no bark
'Egremont Russet'	bark	'Ellison's Orange'	no bark
'Golden Pippin'	bark	'Api rose'	no bark
'Hetlina'	bark	'Braeburn'	no bark
'Ingrid Marie'	bark	'Gala'	no bark
'King David'	bark	'Royal Gala'	no bark
'Robusta 5'	bark	'Royal Gala Ten Hove'	no bark
'Spartan'	bark		

A3.2 Tissue samples collected from a total of 29 apple blocks in the main sampling conducted in spring (M1) and autumn (M2) for endophyte isolation.

Company (Region)	Cultivar	Block[#]	Infection level	Site	Management practice
Kono (Nelson)	'Scilate'	Block 1	high	Site 10	IFP
	'Scilate'	Block 2	low	Site 11	IFP
	'Royal Gala'	Block 3*	low	Site 1	IFP
	'Braeburn'	Block 4*	low	Site 1	IFP
Hoddy (Nelson)	'Royal Gala'	Block 6*	low	Site 2	IFP
	'Royal Gala' (Brookfield-redder)	Block 5	high	Site 2	IFP
	'Scifresh'	Block 7	low	Site 2	IFP
	'Braeburn'	Block 8*	low	Site 2	IFP
Easton (Nelson)	'Braeburn' (Mariri Red)	Block 9	low	Site 3	IFP
	'Royal Gala'	Block 10	low	Site 3	IFP
Burnside (Nelson)	'Braeburn'	Block 11	low	Site 9	IFP
	'Braeburn'	Block 12	high	Site 9	IFP
	'Scifresh'	Block 14	medium	Site 9	IFP
Thomas Brother (Nelson)	'Scifresh'	Block 18	high	Site 12	IFP
	'Braeburn'	Block 20*	low	Site 4	IFP
	'Royal Gala'	Block 21*	low	Site 4	IFP
T&G Global (Nelson)	'Scilate'	Block 23	Low	Site 13	IFP
T&G Global (Hawke's Bay)	'Royal Gala' (Galaxy)	Block 25	low	Site 5	IFP
	'Braeburn'	Block 26	low	Site 5	IFP
	'Scifresh'	Block 27	low	Site 5	IFP
	'Scifresh'	Block 28	high	Site 5	IFP
	'Scilate'	Block 29	high	Site 14	IFP
Mr Apple (Hawke's Bay)	'Royal Gala'	Block 30	low	Site 6	IFP
	'Braeburn'	Block 31	low	Site 6	IFP
	'Scifresh'	Block 32	low	Site 6	IFP
Bostock NZ (Hawke's Bay)	'Royal Gala'	Block 33	low	Site 7	organic
	'Braeburn'	Block 34	low	Site 7	organic
	'Royal Gala'	Block 35	low	Site 8	organic
	'Braeburn'	Block 36	low	Site 8	organic

Blocks followed by * mean they were sampled in both M1 and M2 samplings. The other blocks were only sampled in the M1 sampling.

A3.3 Recipes of media

Synthetic low-nutrient agar (SNA, pH 6.5) (Berg et al., 2005)	**per litre**
KH_2PO_4	1 g
KNO_3	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
Glucose (Scharlau, Scharlab S.L.)	0.2 g
Sucrose	0.2 g
1N NaOH	0.6 ml
Difco™ agar (Difco, Becton, Dickinson and Company, USA)	22 g

Waksman agar (Berg *et al.*, 2002)	**per litre**
Proteose peptone (BD, Becton, Dickinson and Company, USA)	5 g
Glucose (Scharlau, Scharlab S.L.)	10 g
Beef extract (Acumedia, Neogen)	3 g
NaCl (LabServ, Thermofisher Scientific Inc.)	5 g
Difco™ agar (Difco, Becton, Dickinson and Company)*	20 g
pH adjusted to 7.2	

* Waksman broth was made without adding the agar.

A3.4 PCR conditions modification for encoding gene detection

For *phlD* and *phzC*, no band was amplified for any the *Pseudomonas* spp. isolates with the PCR condition shown in the Table 3.5. After PCR conditions were modified to 40 cycles with annealing temperature at 70°C for *phlD* and to 35 cycles for *phzC*, there was also no band detected. For *pltC* detection, false positives were found for all seven *Pseudomonas* spp. isolates. When the PCR conditions were modified to 35 cycles with annealing temperature at 75°C, false positive still occurred. However, the size of the false positive band amplified from all the *Pseudomonas* spp. isolates was smaller than the target band size. The PCR product of isolate 7-208(18)b and gel extraction of the band amplified from isolate 26-785(43)b were selected as representatives for sequencing confirmation and found to be negative. Therefore, they were identified as negative. For gene *hcnBC*, bands were amplified from isolates 7-208(18)b, 27-801(89)b, 31b1 and 31b3. Gel extraction of bands which were similar to the target size were sequenced and confirmed to be negative. For gene *prnC*, multiple bands were obtained from all the *Pseudomonas* spp. The bands with similar size to the target size were extracted from agarose gel and confirmed to be negative by sequencing. After PCR conditions were modified to be 35 cycles with annealing temperature at 68°C, isolate 7-208(18)b was found to be positive. However, it was confirmed to be false positive by sequencing.

A3.5 Description of the 39 fungal morphology groups.

Gf1: Irregular, flat, opaque, black spots are spreading on the colony, with/without yellow fluffy, some isolates with pink edge, agar became orange.

Gf2: Irregular, flat, opaque, yellow fluffy with black spots, agar became orange.

Gf3: Irregular, flat, opaque, yellow fluffy (with some whitish/pinkish/greenish brown fluffy), some isolates with pink edge, agar became orange.

Gf4: Irregular, flat, opaque, brown fluffy, agar became brown.

Gf5: Circular, raised, opaque, pinkish/greenish gray fluffy, pinkish/yellowish brown at the bottom of the colony.

Gf6: Irregular, flat, opaque, pinkish/whitish/greenish brown fluffy.

Gf7: Circular, raised, opaque, white and gray fluffy, agar became orange.

Gf8: Irregular, flat, opaque, pinkish brown, agar became pink.

Gf9: Circular, raised, opaque, white fluffy, brown/green at the bottom of the colony.

Gf10: Filamentous, flat, opaque, white/gray/brown fluffy, dark brown in the middle of the bottom of the colony, agar became dark brown, yellowish white spots on the colony.

Gf11: Circular, flat, opaque, greenish brown, with some raised white fluffy.

Gf12: Circular, raised, opaque, pinkish brown/gray fluffy.

Gf13: Circular, raised, opaque, yellowish brown/white fluffy.

Gf14: Circular, umbonate, opaque, (greenish) brown, rings appear on the front side of the colony.

Gf15: Circular, raised, opaque, wrinkled in the middle, dark green at the bottom of the colony.

Gf16: Circular, raised, opaque, white and gray fluffy, yellowish brown at the bottom of the colony.

Gf17: Circular, umbonate, opaque, yellowish white fluffy.

Gf18: Filamentous, flat, opaque, white fluffy, without brown at the bottom of the colony.

Gf19: Filamentous, flat, translucent, white fluffy, some with greenish brown in the middle of the front side of the colony.

Gf20: Circular, raised, opaque, greenish gray fluffy in the middle of the front side of the colony, light gray fluffy at the edge, yellowish brown at the bottom of the colony.

Gf21: Circular, flat, translucent, (brownish) white fluffy with filamentous rings.

Gf22: Circular, flat, opaque, white fluffy, brown/dark green in the middle of the colony.

Gf23: Circular, flat, opaque, yellowish brown fluffy.

Gf24: Circular, flat, translucent, yellowish white fluffy, green spots appear on some isolates, agar became light yellow.

Gf25: Circular, flat, translucent, yellowish white fluffy, dark green spots appear from the middle to the edge of the colony, agar became yellow.

Gf26: Circular, flat, opaque, greenish gray fluffy.

Gf27: Circular, raised, opaque, yellowish white fluffy in the middle, translucent at the edge, most of isolates wrinkled.

Gf28: Circular, flat, opaque, dark green fluffy in the middle of the colony, white fluffy at the edge.

Gf29: Irregular, convex, opaque, white fluffy, brown at the bottom of the colony, most of isolates are wrinkled.

Gf30: Circular, flat, opaque, brown, some isolates are wrinkled in the middle of the colony.

Gf31: Irregular, raised, opaque, wrinkled, orange fluffy.

Gf32: Yellowish white, smooth, flat, translucent, circular, yeast-like fungi

Gf33: Circular, (yellowish) white fluffy, some isolates are brown at the bottom of the colony.

Gf34: Circular, flat, opaque, white fluffy, agar became pinkish.

Gf35: Irregular, flat, opaque, white fluffy.

Gf36: Circular, flat, opaque, brown fluffy.

Gf37: Circular, flat, opaque, brown fluffy, agar became brown.

Gf38: Irregular, flat, opaque, white, smooth and glistening.

Gf39: Circular, flat, opaque, white and pink fluffy, pink at the bottom of the colony.

A3.6 Sequences of the three largest groups of culturable fungi

> *Stemphylium* sp. (Gf5)

ATTGGGCACCCTACTGATCGAGGTCAAAAGTTGAAAAAAATGTGGTCTTGATGGATGCTCAACCAAGG
CTGATTCAAAGTGCAAGAATTGTGCTGCGCTCCGAAACCAGTAGGTCGGCTGCCAATCATTTTAAGGC
GAGTCTCGTGAGAGACAAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAG
GCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCA
ATTCACACTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAA
AGTTGTAATAATTACATTGTTTACTGACGCTGATTGCAATTACAAAAAGGTTTATGGTTTGGTCCTGG
TGGCGGGCGAACCCGCCCAGGAAACAAGAAGTGCGCAAAAGACATGGGTGAATAATTCAGACAAGCTG
GAGCCCTCACCGAGGTGAGGTCCCAACCCGCTTTCATATTGTGTAATGATCCCTCCGCAGGTTCACCT
ACGGAGACCTTGTTACGACTTTTACTTCCTCTAAATGACCAAG

> *Cladosporium* sp. (Gf11)

ATTGGGATCCTACTGATCCGAGGTCACCTTAGAATGGGGTTGTTTTACGGCGTAGCCTCCCGAACACC
CTTTAGCGAATAGTTTCCACAACGCTTAGGGGACAGAAGACCCAGCCGGTCGATTTGAGGCACGCGGC
GGACCGCGTTGCCCAATACCAAGCGAGGCTTGAGTGGTGAAATGACGCTCGAACAGGCATGCCCCCCG
GAATACCAGGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACT
TATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTAAAAGTTTTAATTTA
TTAATTAAGTTTACTCAGACTGCAAAGTTACGCAAGAGTTTGAAGTGTCCACCCGGAGCCCCCGCCCG
AAGGCAGGGTCGCCCCGGAGGCAACAGAGTCGGACAACAAAGGGTTATGAACATGCCGGTGGTTAGAC
CGGGGTCACTTGTAATGATCCCTCCGCAGGTTCACCTACGGAGACCTTGTTACGACTTTTACTTCCTC
TAAATTGACCAAGA

> *Alternaria* sp. (Gf20)

Isolate 1:
ATGGGGAACCTACCTGATCCGAGGTCAAAAGTTGAAAAAAGGCTTAATGGATGCTAGACCTTTGCTGA
TAGAGAGTGCGACTTGTGCTGCGCTCCGAAACCAGTAGGCCGGCTGCCAATTACTTTAAGGCGAGTCT
CCAGCAAAGCTAGAGACAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAG
GCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCA
ATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAA
AGTTGTAATTATTAATTTGTTACTGACGCTGATTGCAATTACAAAAGGTTTATGTTTGTCCTAGTGGT
GGGCGAACCCACCAAGGAAACAAGAAGTACGCAAAAGACAAGGGTGAATAATTCAGCAAGGCTGTAAC
CCCGAGAGGTTCCAGCCCGCCTTCATATTTGTGTAATGATCCCTCCGCAGGTTCACCTACGGAGACCT
TGTTACGACTTTTACTTCCTCTAAATGACCAAGA

Isolate 2:

GCTCGGGATCCTACCTGATCCGAGGTCAAAGTTGAAAAAAGGCTTAATGGATGCTAGACCTTTGCTGA
TAGAGAGTGCGACTTGTGCTGCGCTCCGAAACCAGTAGGCCGGCTGCCAATTACTTTAAGGCGAGTCT
CCAGCAAAGCTAGAGACAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAG
GCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCA
ATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAA
AGTTGTAATTATTAATTTGTTACTGACGCTGATTGCAATTACAAAAGGTTTATGTTTGTCCTAGTGGT

GGGCGAACCCACCAAGGAAACAAGAAGTACGCAAAAGACAAGGGTGAATAATTCAGCAAGGCTGTAAC
CCCGAGAGGTTCCAGCCCGCCTTCATATTTGTGTAATGATCCCTCCGCAGGTTCACCTACGGAGACCT
TGTTACGACTTTTACTTCCTCTAAATTGACCAAGA

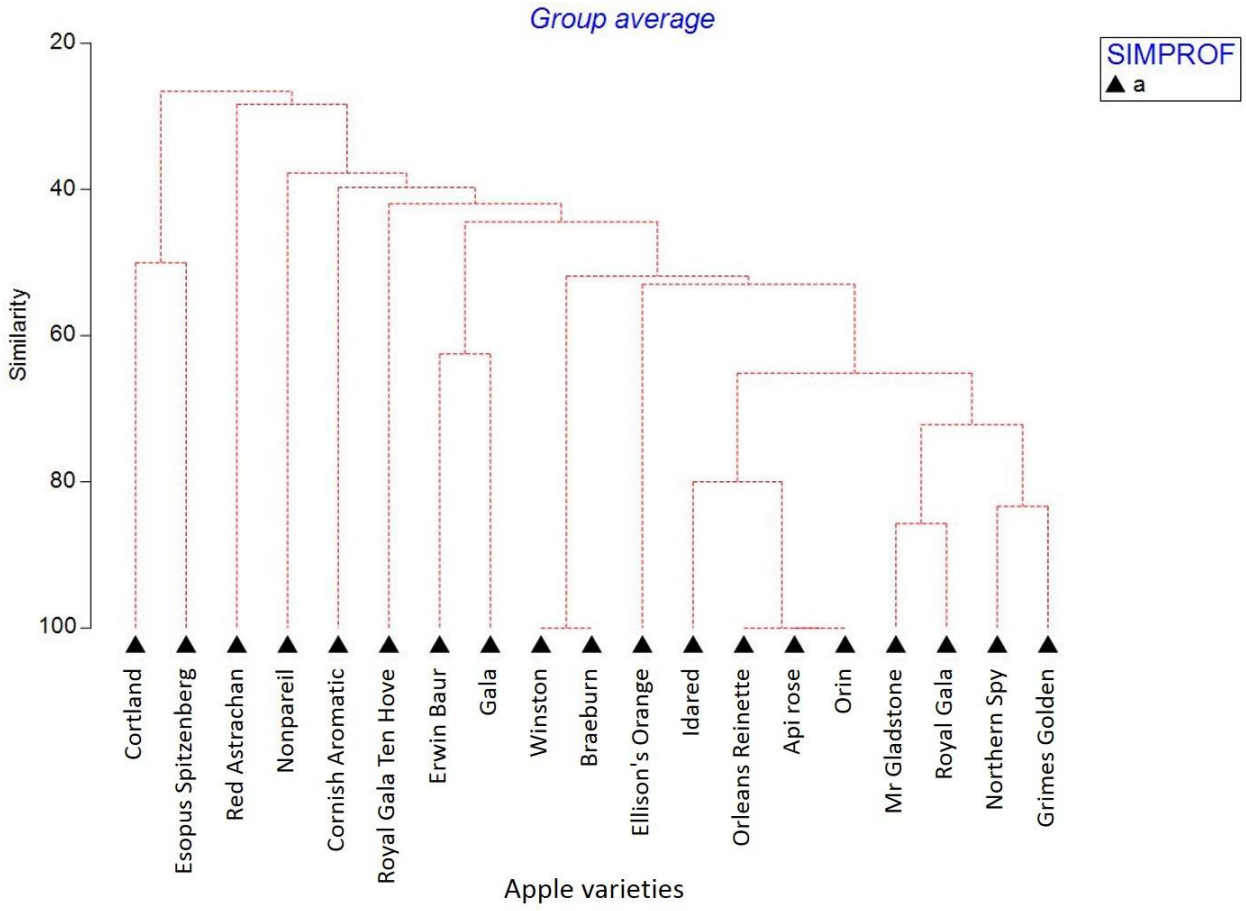
Isolate 3:

GCTTGGGATCCTACCTGATCCGAGGTCAAAGTTGAAAAAAGGCTTAATGGATGCTAGACCTTTGCTGA
TAGAGAGTGCGACTTGTGCTGCGCTCCGAAACCAGTAGGCCGGCTGCCAATTACTTTAAGGCGAGTCT
CCAGCAAAGCTAGAGACAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAG
GCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCA
ATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAA
AGTTGTAATTATTAATTTGTTACTGACGCTGATTGCAATTACAAAAGGTTTATGTTTGTCCTAGTGGT
GGGCGAACCCACCAAGGAAACAAGAAGTACGCAAAAGACAAGGGTGAATAATTCAGCAAGGCTGTAAC
CCCGAGAGGTTCCAGCCCGCCTTCATATTTGTGTAATGATCCCTCCGCAGGTTCACCTACGGAGACCT
TGTTACGACTTTTACTTCCTCTAAATTGACCAAGA

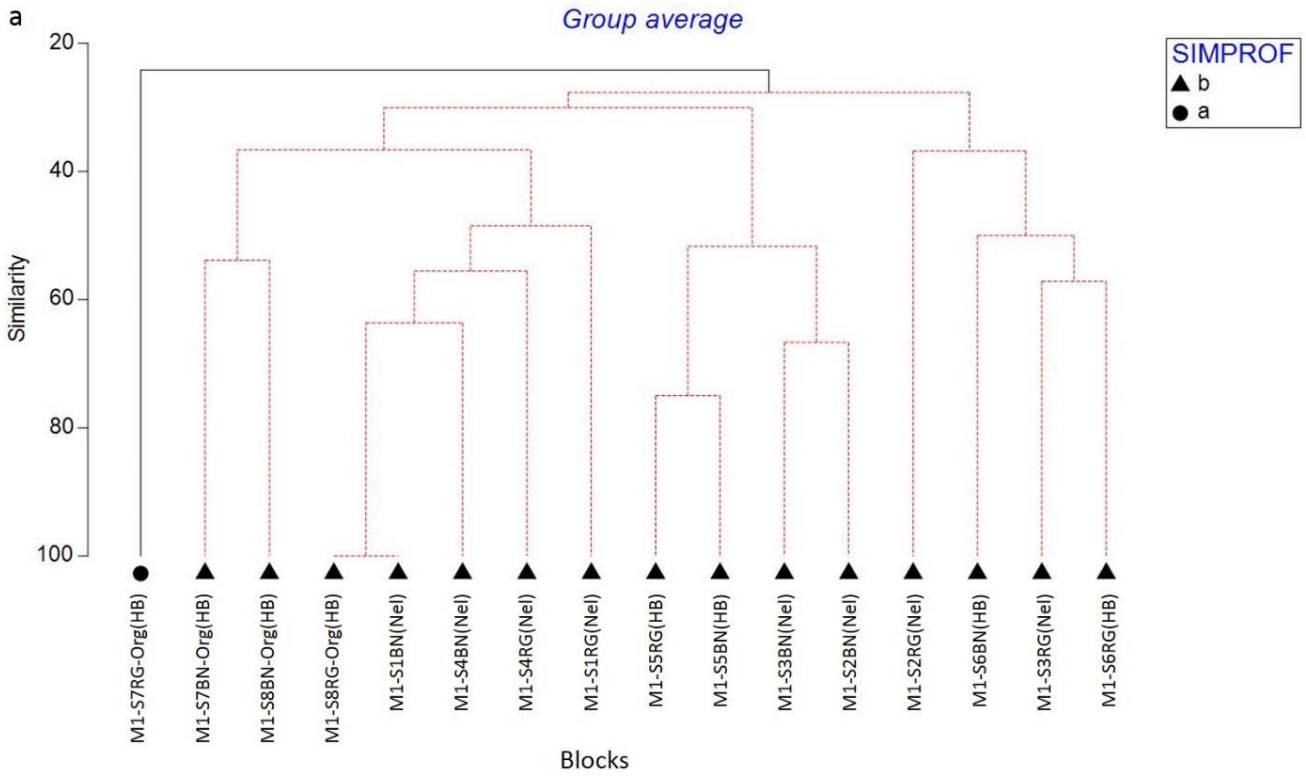
Isolate 4:

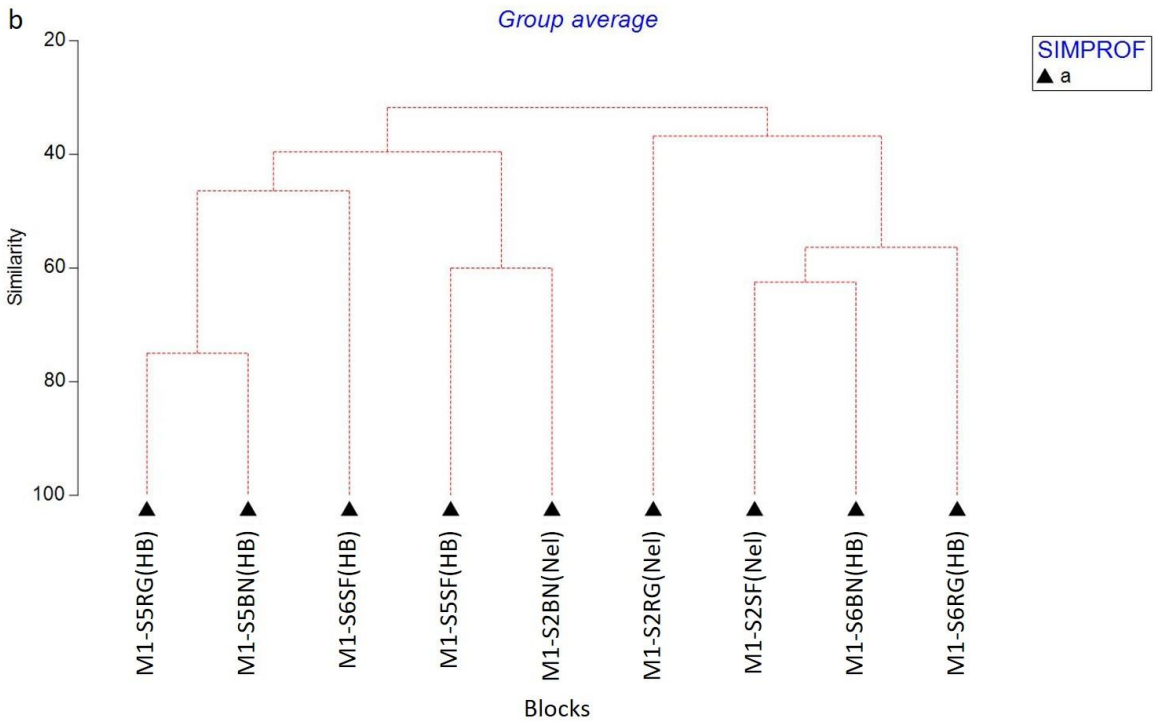
GGCGGGGGACCTACCTGATCGAGGTCAAAGTTGAAAAAAGGCTTAATGGATGCTAGACCTTTGCTGAT
AGAGAGTGCGACTTGTGCTGCGCTCCGAAACCAGTAGGCCGGCTGCCAATTACTTTAAGGCGAGTCTC
CAGCAAAGCTAGAGACAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGG
CATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAA
TTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAA
GTTGTAATTATTAATTTGTTACTGACGCTGATTGCAATTACAAAAGGTTTATGTTTGTCCTAGTGGTG
GGCGAACCCACCAAGGAAACAAGAAGTACGCAAAAGACAAGGGTGAATAATTCAGCAAGGCTGTAACC
CCGAGAGGTTCCAGCCCGCCTTCATATTTGTGTAATGATCCCTCCGCAGGTTCACCTACGGAGACCTT
GTTACGACTTTTACTTCCTCTAATTGACCAAGA

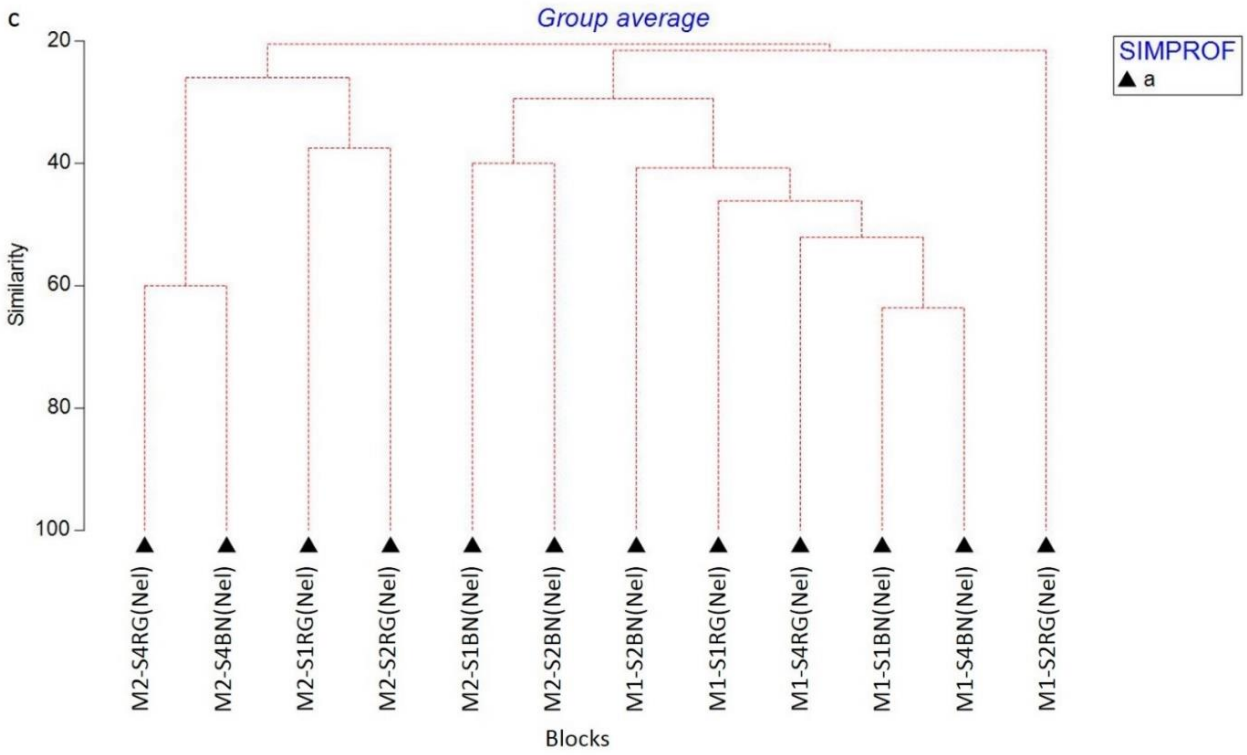
A3.7 SIMPROF profile using group average cluster analysis of the endophytic culturable fungi groups similarities from the presence and absence data of each morphotype recovered from the 15 heritage varieties and four commercial varieties ('Royal Gala Ten Hove', 'Gala', 'Braeburn' and 'Royal Gala') with bark removed for isolation.

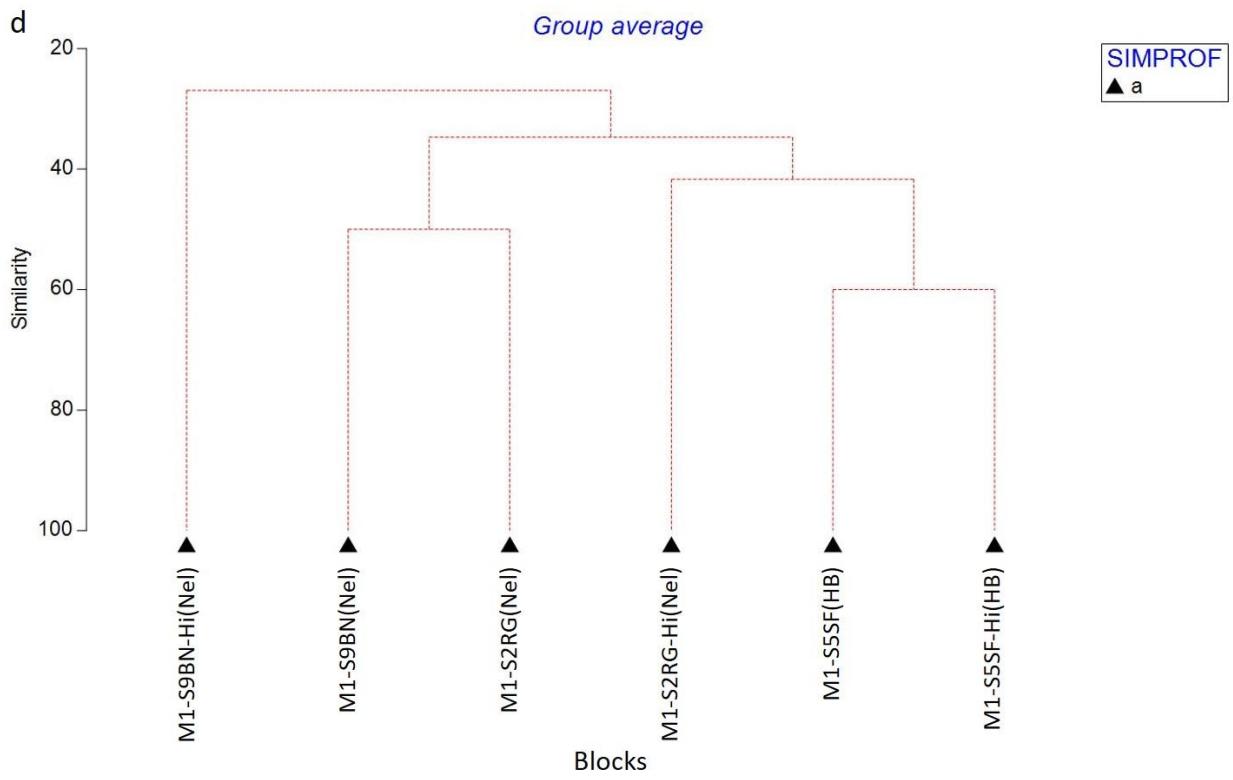


A3.8 SIMPROF profile using group average cluster analysis of the endophytic culturable fungi groups similarities from the presence and absence data of each morphotype from the main sampling conducted in spring (M1) and/or main sampling conducted in autumn (M2) affected by a) region, b) variety, c) season and d) infection level. S means site. RG, BN and SF mean 'Royal Gala', 'Braeburn' and 'Scifresh', respectively. Org means organic. Hi means high European canker infection block. Without Org/Hi means IFP managed orchards/low European canker infection block. Nel and HB mean Nelson and Hawke's Bay, respectively (solid line indicates significant difference at $p = 0.05$).




b
Group average
SIMPROF
a
Similarity
20
40
60
80
100
M1-S5RG(HB)
M1-S5BN(HB)
M1-S6SF(HB)
M1-S5SF(HB)
M1-S2BN(Nel)
M1-S2RG(Nel)
M1-S2SF(Nel)
M1-S6BN(HB)
M1-S6RG(HB)
Blocks


c
Group average
SIMPROF
a
Similarity
20
40
60
80
100
M2-S4RG(Nel)
M2-S4BN(Nel)
M2-S1RG(Nel)
M2-S2RG(Nel)
M2-S1BN(Nel)
M2-S2BN(Nel)
M1-S2BN(Nel)
M1-S1RG(Nel)
M1-S4RG(Nel)
M1-S1BN(Nel)
M1-S4BN(Nel)
M1-S2RG(Nel)
Blocks


d
Group average
SIMPROF
a
Similarity
20
40
60
80
100
M1-S9BN-Hi(Nel)
M1-S9BN(Nel)
M1-S2RG(Nel)
M1-S2RG-Hi(Nel)
M1-S5SF(HB)
M1-S5SF-Hi(HB)
Blocks

A3.9 Identification of the 18 selected antagonistic bacterial isolate showing inhibitory activity to *Neonectria ditissima* ICMP14417 based on sequencing of the 16S rRNA.

Bacterial isolates	Designated code	Region origin	Variety and tissue	Season origin	Representatives of the closest relatives in GenBank (Accession no.)	Identities
42-1206(19)b	*Bacillus* sp. 1	Nelson	Braeburn, leaf	Autumn (M2)	*Bacillus subtilis* strain JK-1 (DQ846632.1)	843/843(100%)
					Bacillus velezensis strain LAB1 (MK263025.1)	842/843(99%)
					Bacillus amyloliquefaciens strain PgBE240 (MH144310.1)	842/843(99%)
R1GS-12b	*Bacillus* sp. 2	Lincoln University	Royal Gala, stem	Autumn (LU)	*Bacillus amyloliquefaciens* strain HR62 (JF700435.1)	746/747(99%)
					Bacillus siamensis strain B268 (KY129717.1)	745/747(99%)
					Bacillus subtilis strain ZJ2 (KY121111.1)	745/747(99%)
					Bacillus velezensis strain Tr02M (MH210868.1)	745/747(99%)
R3L-1b	*Bacillus* sp. 3	Lincoln University	Royal Gala, leaf	Autumn (LU)	*Bacillus velezensis* strain LAB1 (MK263025.1)	658/658(100%)
					Bacillus subtilis strain QB61 (CP029461.2)	658/658(100%)
					Bacillus amyloliquefaciens strain PgBE240 (MH144310.1)	658/658(100%)
					Bacillus tequilensis strain K34.2 (MH889140.1)	658/658(100%)
26-771(23)b	*Bacillus* sp. 4	Hawke's Bay	Braeburn, stem	Spring (M1)	*Bacillus thuringiensis* strain 263XG9 (KF818641.1)	1410/1418(99%)
					Bacillus oryzaecorticis strain WJB155 (KU877673.1)	1409/1418(99%)
					Bacillus cereus strain f24 (KP411923.1)	1409/1418(99%)
					Bacillus toyonensis strain MCCC 1A06937 (KJ812470.1)	1407/1415(99%)

A3.9 Continued

Bacterial isolates	Designated code	Region origin	Variety and tissue	Season origin	Representatives of the closest relatives in GenBank (Accession no.)	Identities
21-606(28)b	*Bacillus* sp. 5	Nelson	Royal Gala, leaf	Spring (M1)	*Bacillus cereus* strain M3 (JF836883.1)	1417/1418(99%)
					Bacillus thuringiensis strain Xmb014 (KT986144.1)	1416/1418(99%)
					Bacillus toyonensis strain CSR_25 (KX035026.1)	1416/1418(99%)
					Bacillus wiedmannii strain FSL W8-0169 (KU198626.1)	1416/1418(99%)
R3L-6b	*Bacillus* sp. 6	Lincoln University	Royal Gala, leaf	Autumn (LU)	*Bacillus thuringiensis* serovar israelensis (AY461762.2)	765/785(97%)
					Bacillus toyonensis strain BNS 102 (KX458036.1)	762/783(97%)
					Bacillus cereus strain CMCC P0011 (CP011153.1)	762/783(97%)
41-1183(6)b	*Bacillus* sp. 7	Nelson	Royal Gala, stem	Autumn (M2)	*Bacillus horneckiae* strain MER_86 (KT719661.1)	651/651(100%)
					Bacillus firmus (DQ355388.1)	651/651(100%)
					Bacillus foraminis strain FV1 (JQ867498.1)	650/651(99%)
41-1183(7)b	*Bacillus* sp. 8	Nelson	Royal Gala, stem	Autumn (M2)	*Bacillus horneckiae* strain MER_86 (KT719661.1)	659/659(100%)
					Bacillus firmus (DQ355388.1)	657/657(100%)
					Bacillus foraminis strain FV1 (JQ867498.1)	659/659(100%)
41-1182(4)b	*Bacillus* sp. 9	Nelson	Royal Gala, leaf	Autumn (M2)	*Bacillus horneckiae* strain JA-1 (KU297171.1)	658/659(99%)
20-579(18)b	*Pseudomonas* sp. 1	Nelson	Braeburn, stem	Spring (M1)	*Pseudomonas orientalis* strain PE38 (KJ127247.1)	1396/1397(99%)
					Pseudomonas fluorescens strain DDEN03 (KR234045.1)	1394/1397(99%)

A3.9 Continued

Bacterial isolates	Designated code	Region origin	Variety and tissue	Season origin	Representatives of the closest relatives in GenBank (Accession no.)	Identities
7-208(18)b	*Pseudomonas* sp. 2	Nelson	Scifresh, stem	Spring (M1)	*Pseudomonas lurida* strain MYb11 (CP023272.1)	748/751(99%)
					Pseudomonas fluorescens strain L228 (CP015639.1)	748/751(99%)
					Pseudomonas tolaasii strain 47 (JX417439.1)	748/751(99%)
39-1143(30)b	*Pseudomonas* sp. 3	Nelson	Royal Gala, stem	Autumn (M2)	*Pseudomonas koreensis* strain PgBE56 (MH144278.1)	603/603(100%)
					Pseudomonas fluorescens strain Pf275 (CP031648.1)	603/603(100%)
					Pseudomonas corrugata MAFF 301669 (LC333822.1)	603/603(100%)
27-801(89)b	*Pseudomonas* sp. 4	Hawke's Bay	Scifresh, leaf	Spring (M1)	*Pseudomonas veronii* strain Azi20 (KJ726603.1)	683/683(100%)
					Pseudomonas synxantha strain LMG 2190 (LT629786.1)	682/683(99%)
					Pseudomonas libanensis strain BS2975 (LT629699.1)	682/683(99%)
					Pseudomonas extremaustralis strain IB-K13-1A (LT617886.1)	682/683(99%)
					Pseudomonas panacis strain NU03 (KX187322.1)	682/683(99%)
					Pseudomonas fluorescens strain LBUM636 (CP012400.1)	682/683(99%)
					Pseudomonas brenneri strain LMTZ064-90 (KU750791.1)	682/683(99%)

A3.9 Continued

31b1	*Pseudomonas* sp. 5	Hawke's Bay	Grimes Golden, stem	Spring (HBHV)	*Pseudomonas azotoformans* strain MUT140 (KT751328.1)	559/562(99%)
					Pseudomonas fluorescens strain A531 (KU312049.1)	558/561(99%)
					Pseudomonas rhodesiae strain BS2777 (LT629801.1)	560/564(99%)
					Pseudomonas fulva strain SW20 (KT363045.1)	560/564(99%)
31b3	*Pseudomonas* sp. 6	Hawke's Bay	Grimes Golden, stem	Spring (HBHV)	*Pseudomonas fluorescens* strain K11 (KT767975.1)	1398/1399(99%)
					Pseudomonas rhodesiae (HE716938.1)	1398/1399(99%)
26-785(43)b	*Pseudomonas* sp. 7	Hawke's Bay	Braeburn, leaf	Spring (M1)	*Pseudomonas poae* strain ICE349 (KX588598.1)	1399/1401(99%)
21-615(16)b	*Pseudomonas* sp. 8	Nelson	Royal Gala, stem	Spring (M1)	*Pseudomonas syringae* strain PDD-50b-5 (KR922071.1)	1393/1398(99%)
					Pseudomonas amygdali pv. *tabaci* strain BRST-1 (KR476390.1)	1394/1399(99%)
					Pseudomonas congelans strain XjGEB-29 (JQ320090.1)	1394/1399(99%)
26-771(6)b	*Pseudomonas* sp. 9	Hawke's Bay	Braeburn, stem	Spring (M1)	*Pseudomonas syringae* strain PDD-32b-19 (KR922052.1)	1367/1368(99%)

A3.10 Identification of the 18 selected endophytic fungal isolate showing inhibitory activity to *Neonectria ditissima* ICMP14417 based on sequencing of the ITS region.

Isolate no.	Designated code	Variety origin	Tissue origin	Morphology group	Representatives of the closest relatives in GenBank (Accession no.)	Identities
2-51f	*Biscogniauxia* sp. 1	Scilate	green stem	Gf19	*Biscogniauxia* sp. 2 ICMP 18793 (JN225897.1)	570/572(99%)
10-283(1)f*	*Biscogniauxia* sp. 2	Royal Gala	green stem	Gf10	*Biscogniauxia* sp. 1 ICMP 18828 (JN225898.1)	727/730(99%)
2-66f	*Biscogniauxia* sp. 3	Scilate	leaf	Gf10	*Biscogniauxia* sp. 1 ICMP 18828 (JN225898.1)	747/748(99%)
20-594f	*Biscogniauxia* sp. 4	Braeburn	leaf	Gf10	*Biscogniauxia* sp. 2 ICMP 18793 (JN225897.1)	600/603(99%)
14-X(2)f	*Diaporthe* sp. 1	Scifresh	unknown	Gf21	*Diaporthe viticola* culture-collection ICMP:16419 (KC145904.1)	567/568(99%)
					Phomopsis sp. I405 (GU584955.1)	567/568(99%)
					Diaporthe cynaroidis culture-collection CBS:122676 (EU552122.1)	565/568(99%)
					Diaporthe salicicola BRIP 54825 (NR_137106.1)	563/568(99%)
					Diaporthe rudis strain CBS 100170 (KC343230.1)	548/549(99%)
1-38f	*Chaetomium* sp. 1	Scilate	leaf	Gf19	*Chaetomium* cf. *cochliodes* MZ-2011 strain CCM F-232 (KT895345.1)	532/532(100%)
					Chaetomium globosum strain A95 (KT898661.1)	531/532(99%)
1-35f	*Chaetomium* sp. 2	Scilate	green stem	Gf25	*Chaetomium* cf. *cochliodes* MZ-2011 strain CCM F-232 (KT895345.1)	532/532(100%)
					Chaetomium globosum strain A95 (KT898661.1)	531/532(99%)
2-57f	*Chaetomium* sp. 3	Scilate	leaf	Gf25	*Chaetomium globosum* isolate BK250A (KU702697.1)	318/320(99%)
					Chaetomium graminiforme strain CBS 506.84 (KT214584.1)	318/320(99%)
					Chaetomium elatum strain P10-17a (KT354986.1)	318/320(99%)

A3.10 Continued

Isolate no.	Designated code	Variety origin	Tissue origin	Morphology group	Representatives of the closest relatives in GenBank (Accession no.)	Identities
35-1049(2)f	*Epicoccum* sp. 1	Royal Gala	leaf	Gf1	*Epicoccum nigrum* strain G392 (KR094461.1)	526/527(99%)
34-1029(1)f	*Epicoccum* sp. 2	Braeburn	leaf	Gf2	*Epicoccum nigrum* strain G392 (KR094461.1)	522/522(100%)
36-1073(1)f	*Epicoccum* sp. 3	Braeburn	leaf	Gf2	*Epicoccum nigrum* strain G392 (KR094461.1)	529/530(99%)
4-105(2)f	*Epicoccum* sp. 4	Braeburn	leaf	Gf3	*Epicoccum nigrum* strain 127PRJ (KU319072.1)	526/526(100%)
					Ceratocystis paradoxa strain UAMH 8784 (KC305160.1)	526/526(100%)
					Glonium pusillum culture-collection CBS:119348 (EU552134.1)	526/526(100%)
					Saperda carcharias isolate 24 (KJ702040.1)	524/524(100%)
34-1026(3)f	*Epicoccum* sp. 5	Braeburn	leaf	Gf3	*Epicoccum nigrum* strain G392 (KR094461.1)	527/527(100%)
3-73f	*Neoseptophoma* sp./ *Leptosphaeria* sp./ *Coniothyrium* sp.	Royal Gala	leaf	Gf8	*Neosetophoma clematidis* voucher MFLUCC 13-0734 (KP744450.1)	536/541(99%)
					Leptosphaeria sp. E-000535665 (JN545785.1)	518/524(99%)
					Neosetophoma italica culture-collection MFLU:14 C0809 (KP711356.1)	531/551(96%)
					Coniothyrium sp. 97TB (KF573980.1)	504/510(99%)
36-1072f	*Phlyctema* sp. 1	Braeburn	woody stem	Gf29	*Phlyctema vagabunda* isolate PS-21 (KT923788.1)	508/508(100%)
					Neofabraea alba strain ID04 (KJ396077.1)	508/508(100%)
36-107Xf	*Phlyctema* sp. 2	Braeburn	unknown	Gf29	*Phlyctema vagabunda* isolate PS-21 (KT923788.1)	525/525(100%)
					Neofabraea alba strain CR4 (KJ396075.1)	525/525(100%)
20-578f	Unidentified	Braeburn	leaf	Gf18	*Xylariaceae* sp. TA1-4-1 (JX914484.1)	598/668(90%)
6-176f	*Penicillium* sp. 1	Royal Gala	woody stem	Gf10	*Penicillium ochrochloron* clone Fl19 (KX082933.1)	434/435(99%)

* The isolate 10-283(1)f was contaminated.

A3.11 Sequences of the 18 antagonistic bacterial isolates and 18 antagonistic fungal isolates

> *Bacillus* sp. 21-606(28)b

TGCAAGTCGAGCGAATGGATTAGAGAGCTTGCTCTTAAGAAGTTAGCGGCGGACGGGTGAGTAACA
CGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTT
TGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCAT
TAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATG
GACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGT
TAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAA
AGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGG
AAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAG
ATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCG
TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGA
GGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAG
GCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGG
AGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTG
CTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCA
GTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATG
CCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCC
GAAGTCGGTGGGGTAACCTTTATGGAGCCAGC

> *Bacillus* sp. 26-771(23)b

TGCAGTCGAGCGAATGGATTAGAGAGCTTGCTCTCATGAAGTTAGCGGCGGACGGGTGAGTAACAC
GTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTT
GAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG
ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTT
AGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAA
GCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA
AACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCCATGTGTAGCGGTGAAATGCGTAGAG
ATATGGAGGAACACCAGTTGGCGAAGGCGACTTTTCTGGTCTGTAAACTGACACTGAGGCGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCCTGGTAGTCCACCGCCGTAAACGATGAGTGCTAAGTG
TTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCC
GCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCC
TTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA
CACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCG
TTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATC

AGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTA
ACACCCGAAGTCGGTGGGGTAACCTTTATGGA

> *Bacillus* sp. R3L-1b

ACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATT
AAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTG
ACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATT
CAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCAT
CATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCG
AGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAG
CTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCC

> *Bacillus* sp. R3L-6b

GGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTGAGAGCTTGC
TCTCAAGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAAC
TCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACTGCATGGTTCGAAATTGAAAGGCGGCT
TCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG
CAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAG
TGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTG
GCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGTGGCAAGCGTTATCCGGAATTATTGGGGGAAAGCGCGCGCGGGTGGTTTCTTAAGTCTGATGTG
AAAAGCCCACGGTTCAACCCGGGGAGGGTCATTTGGAAAACTGGGAGACTTGAGTGCAGAAGAGGA
AAGTGGAATTCCATGTGTTAGCGGTGAAAATGCGTAGAGATATGGAGGAACACCAGTGGCCGAAGG
CGACTTTTCTGGGCCTGGTAACTGACACTGAGGGGCCGAAAGCGTGGGGGAGCAAAACAG

> *Bacillus* sp. R1GS-12b

ATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTG
AACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTA
GCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA
CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTA
GGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAG
GGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAA
ACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGAT
GTGGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGT
GGGGAGCGAACAGGGATTAGA

> *Bacillus* sp. 41-1183(7)b

CGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGC
ACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAA

TCCTAGAGATAGGACGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCA
GTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCA
TGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAGACCGCGAG
GTTTAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCC
GGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTGGAGCCAGCCGCCT

> *Bacillus* sp. 41-1183(6)b

CGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTA
AGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAA
GCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGA
CAATCCTAGAGATAGGACGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCAT
TCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCA
TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAGACCGC
GAGGTTTAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAA
GCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACAC
CGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTGGA

> *Bacillus* sp. 41-1182(4)b

GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAA
ACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA
TCCTCTGACAATCCTAGAGATAGGACGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTT
GCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
TCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGC
AAGACCGCGAGGTTTAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCC
TGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTT

> *Bacillus* sp. 42-1206(19)b

GGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAAATTCCACGTGTAGCG
GTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGC
TGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG
GAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAG
GACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAA
GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACC
TGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCC
ACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAA
TCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGA
GAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCC

> *Pseudomonas* sp. 20-579(18)b

AGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAAAGCCTAGGAAT
CTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAA
GCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAA
TGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACA
CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGC
CATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTA
CCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCG
CGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGT
TAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTGT
GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGG
CGAAGGCGACCACCTGGACCAACACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGC
GCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGC
CTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAGCATTGAGACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCT
TAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGG
GATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGA
GGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCA
ACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCT
TCGGGAGGACG

> *Pseudomonas* sp. 21-615(16)b

ATGCAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTGGCGAGCGGCGGACGGGTGAGTAATGCCT
AGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCCTACGG
GAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG
AGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACT
GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGA
TCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGG
CAGTTACCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAG
CAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTG
GTTTGTTAAGTTGAATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTA
GAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACAC
CAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCCGGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCT
TTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA
TGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCT
TACCCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACATTGAGACA
GGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAAC
CCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGT
CGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCG
CAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTA
GTCTAACCTTCG

> *Pseudomonas* sp. 26-771(6)b

GTACCTGGTGGCGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAAC
GCTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGC
GCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCC
GTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG
TCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAATACGTATCTGTTTTGAC
GTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGC
GTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGAATGTGAAATCCCCG
GGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCC
TGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGA
TACTGACACTGAGGTGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGA
CCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTC
CAGAGATGGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTAAGGT
GGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG
CCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTG
GAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGA
ATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
TCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCG

> *Pseudomonas* sp. 26-785(43)b

TGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAG
GAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGA
GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGG
GTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATC
CAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCA
GTTACCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCA
GCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGT
TTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGA
GTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCA
GTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAAGCCTTGAGCTTTTA
GTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTG
CTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTT
GTCCTTAGTTACCAGCACGTCATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGT
ACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGT
CTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACG
TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCT
AACCTTCGGGAGGAC

> *Pseudomonas* sp. 31b3

TGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAG
GAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGA
GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGG
GTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATC
CAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCC
GTTACCTAATACGTGATGGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCA
GCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGT
TTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGA
GTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCA
GTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACCGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTA
GTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTG
CTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTT
GTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGT
ACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGT
CTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACG
TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCT
AACCTTCGGGGGGGAC

> *Pseudomonas* sp. 7-208(18)b

GTCGTCAGCTCGTGTCGTAGATGTTGGGTTAAGTCCCGTAACGAGCCAACCCTTGTCCTTAGTTAC
CAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
TCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTCC
AAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACT
GCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAAACTTCGGGAGG
ACGGTT

> *Pseudomonas* sp. 27-801(89)b

CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTG
AGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAAC
TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGA
GACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCG
CAACCCTTGTCCTTAGTTACCAGCACGTCATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAA
TGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGTAGTCCGG
ATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGG
TGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTA
GCTAGTCTAACCTTCGGGGGGAC

> *Pseudomonas* sp. 31b1

CCGCAAAGGTTAAAACTCAAATGAATTGGACGGGGGGCCGCACAAGCGGTGGAGCATGTGGTTTAA
TTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGT
GCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGG
CTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAA
AACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGC
GAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGT
GGGTTGCACCAGAAGTAGCTAGTCTAACTTCGGGG

> *Pseudomonas* sp. 39-1143(30)b

GAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAA
CTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACATTG
AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGC
GCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAAC
CGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACA
ATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCACAAAACCGATCGTAGTCCG
GATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGT
AGCTAGTCT

> *Chaetomium* sp. 1-35f

AAGGTGGTTTAACGGCCGGAACCCGCAGCACGCCCAGAGCGAGATGTATGCTACTACGCTCGGTGT
GACAGCGAGCCCGCCACTGCTTTTCAGGGCCTGCGGCAGCCGCAGGTCCCCAACACAAGCCCGGGG
CTTGATGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTT
CAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCA
TCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGACTTATTCAGTACAGAAGACTCAGAGA
GGCCATAAATTATCAAGAGTTTGGTGACCTCCGGCGGGCGCCCGCGGTGGGGCCCAGGGGCGCCCG
GGGGGTAAACCCCGGGGCCGCCCGCCGAAGCAACGGTTTAGGTAACGTTCACAATGGTTTAGGGAG
TTTTGCAACTCTGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTTTTACTTC
CTCT

> *Chaetomium* sp. 1-38f

AAGGTGGTTTAACGGCCGGAACCCGCAGCACGCCCAGAGCGAGATGTATGCTACTACGCTCGGTGT
GACAGCGAGCCCGCCACTGCTTTTCAGGGCCTGCGGCAGCCGCAGGTCCCCAACACAAGCCCGGGG
CTTGATGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTT
CAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCA
TCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGACTTATTCAGTACAGAAGACTCAGAGA
GGCCATAAATTATCAAGAGTTTGGTGACCTCCGGCGGGCGCCCGCGGTGGGGCCCAGGGGCGCCCG
GGGGGTAAACCCCGGGGCCGCCCGCCGAAGCAACGGTTTAGGTAACGTTCACAATGGTTTAGGGAG
TTTTGCAACTCTGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTTTTACTTC
CTCT

> *Chaetomium* sp. 2-57f

AGGTGGTTTAACGGCCGGAACCCGCAGCACGCCCAGAGCGAGATGTATGCTACTACGCTCGGTGTG
ACAGCGAGCCCGCCACTGCTTTTCAGGGCCTGCGGCAGCCGCAGGTCCCCAACACAAGCCCGGGGC
TTGATGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTC
AAAGATTCGATGATTCACTGAATTCTGAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATC
GATGCCAGAACCAAGAAATCCGTTGTTGAAAGTTTTGACTTATTCAGTACAGAAG

> *Biscogniauxia* sp. 2-51f

GGTCACCAGTAAAAAATATAGGGGGTTTTACGGCAGGACCTAGGCCGGCTGCAGAAGCGAGGGTAT
GATTACTACGCTTAGAGCACGAACTAGCTCCGCCACTATATTTGAGGAGTTACGTCTCCGTAAGCT
CCCAACGTCAAGCAAATAGGGCTTGGGGGTTGAAATGACGCTCGAACAGGCATGCCTAACAGAATA
CTATTAGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTAT
CGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTTAT
TTGTATAATAGACTCAGAGATACAGTTGTAAAACAGAGTTTGGGGGTCCTTCGGCAGGCCTGTTTG
CGTGCTGCAGGGTAGGCGACGCCAACTCCAGGGTAGCTACTCCAGGGTAGCTGCTCCAGGGTAGGC
GGTCCGGCCGCGCATCACGACCTGCCGAGGCAACAAAAGGTATGTTCACATGGGTTTGGAGTTTGT
AATTAACTCGCTAATGATCCCTCCGCTGGTTCACCAACGGAGA

> *Biscogniauxia* sp. 2-66f

ATCCGAGGTCAACCAGAATAAAATTTAGGGGGGGTTTTACGGCAGGATATAGGGCAGCATCAGGAG
CGAGAGAAAAAAGAATTACTACGCTCAGAGCATGACCTAGCTCCGCCACTAAATTTAAGGAGTTAC
AGCAGCTGTAAGCTCCCAACGTCAAGCAAATAGGGCTTGGGGGTTGAAATGACGCTCGAACAGGCA
TGCCTAACAGAATACTATTAGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAA
TTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGA
AAGTTTTAACTTATTTGTATAATTCGCTCAGAGATACAGTATAATTTCAGAATTTAGGGGTCCTTC
GGCAGGCCTTGTAGCGTGCTACAGGGTAGGCGCTGCACTGCTGGTAGCTTTCCTACTCCAGGGTAG
CCTCTCTCCAGAGTGAGAGCTAAGCTCCGCACCAGGGCAGCTCCTCTCCAGGGTAAATTCAGCCGT
AGCGTTCATTACTCCAGGGTAGCTTCTCTCCAGCGCGCAAGCCCAACTCTACTCCAGGGTAGCTCC
TCTCCAGGGTAGGTCCGCTGCAGGGCAGCTTAGCCGTGCCGTATAGCACGGCCTGCCGAGGCAACA
GTAGGTAAGTTCACATGGGTTTGGAGTTTATAATTAACTCGCTAATGATCCCTCCGCTGGTTCACC
AACGGAGACCTTGTTACGACTTTTACTTCCT

> *Biscogniauxia* sp. 10-283(1)f

TGATCGAGGTCACCAGAATAAAATTTAGGGGGGGTTTTACGGCAGGATATAGGGCAGCATCAGGAG
CGAGAGAAAAAAGAATTACTACGCTCAGAGCATGACCTAGCTCCGCCACTAAATTTAAGGAGTTAC
AGCAGCTGTAAGCTCCCAACGTCAAGCAAATAGGGCTTGGGGGTTGAAATGACGCTCGAACAGGCA
TGCCTAACAGAATACTATTAGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAA
TTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGA
AAGTTTTAACTTATTTGTATAATTCGCTCAGAGATACAGTATAATTCAGAATTTAGGGGTCCTTCG
GCAGGCTTTGTAGCGTGCTACAGGGTAGGCGCTGCACTGCTGGTAGCTTTCCTACTCCAGGGTAGC
CTCTCTCCAGAGTGAGAGCTAAGCTCCGCACCAGGGCAGCTCCTCTCCAGGGTAAATTCAGCCGTA
GCGTTCATTACTCCAGGGTAGCTTCTCTCCAGCGCGCAAGCCCAACTCTACTCCAGGGTAGCTCCT
CTCCAGGGTAGGTCCGCTGCAGGGCAGCTTAGCCGTGCCGTATAGCACGGCCTGCCGAGGCAACAG
TAGGTAAGTTCACATGGGTTTGGAGTTTATAATTAACTCGCTAATGATCCCTCCGCTGGTTCACCA
AC

> Unidentified 20-578f

ATCCGAGGTCACCAGTATAAAATTTGGGGGTTTCACGGCAGGATACAGGACAGCGTCAGAAGCGAG
AATAAAATTACTACGCTCAGAGCATGACCCGGCTCCGCCACTAAATTTAAGGAGTTACAGCAGCTG
TAAGTTCCCAACGTCAAGCAAATAGGGCTTGGGGGTTGAAATGACGCTCGAACAGGCATGCCTAAC
AGAATACTATTAGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATT
ACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTA
ACTTATTTGTATAATTTACTCAGAGATACAGTTACAAAACAGAGTTTAGGGGTCCTTCGGCAGGCC
TGGTTGTGTGCTATAGGGTAGGTCGCTGGTCTGCTCGAAAGCTGTCCGTACTCTAAAGGTGGCCTC
TCTCCAAAGTTCTTACACTCCAGGGTAGCTCCTATTTAGGGTATGTCAGCTACAGGGTAGCTTCCA
GTACCGTATAACACGGCCTGCCGAGGCAACAATAGGTAAGTTCACATGGGTTTGGAGTTTGTAATT
AACTCGCTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTTTTACTTCCTCTA

> *Biscogniauxia* sp. 20-594f

CCTGATCGAGGTCACCAGTAAAAAATATAGGGGGTTTTACGGCAGGACCTAGGCCGGCTGCAGAAG
CGAGGGTATGATTACTACGCTTAGAGCACGAACTAGCTCCGCCACTATATTTGAGGAGTTACGTCT
CCGTAAGCTCCCAACGTCAAGCAAATAGGGCTTGGGGGTTGAAATGACGCTCGAACAGGCATGCCT
AACAGAATACTATTAGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCAC
ATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTT
TTAACTTATTTGTATAATAGACTCAGAGATACAGTTGTAAAACAGAGTTTGGGGGTCCTTCGGCAG
GCCTGTTTGCGTGCTGCAGGGTAGGCGACGCCAACTCCAGGGTAGCTACTCCAGGGTAGCTGCTCC
AGGGTAGGCGGTCCGGCCGCGCATCACGACCTGCCGAGGCAACAAAAGGTATGTTCACATGGGTTT
GGAGTTTGTAATTAACTCGCTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTT
TTACTTCCTCTA

> *Neosetophoma* sp. 3-73f

GGCTTACTGGACGCCAGCATTACAGGCATGACTCGCAAAATGTGCTGCGCTTCAATACCAAAACAC
TGGCTGCCAATTGCTTTAAGGCGAGTCCACACACGGCGGAGTGGACAAACACCCAACACCAAGCAG
AGCTTGAGGGTACAAATGACGCTCGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCG
TTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTT
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TGAAAATTAAAAAGGTTATAGTGTTGTCCAGCCGGCGGGGGTTAGCCGCCGAGGAAACATGAGTGC
GCAAAAGACAAGGGTACAGACAGAGGGTCGTGCTGCTCATCAGTAGTGATACTATACAGGCTGATA
GATCCCCCTAACAGTAGCAAGCTACTGAATGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCT
TGTTACGACTTTTACTTCC

> *Phlyctema* sp. 36-107Xf

AGGTCAACCTGTAAAAATGGGGGGTTCTGGCGAGCATCCACCGGGCATCCAGAGCGAGAGATTTAC
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CAAGCAGAGCTTGAGGGTTGTAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAAGGGGCGCA
ATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTG
CGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTATTATATAGTACTCA
GACGACACTAAATTTCAGAGTTTGGGGTCCTCTGGCGGGCGCTCACCAGCCGGAGCCGGTGGCCGA
GGCGGGCCCGCCAAAGCAACAAAGGTGTGATGACACGGGTGGGAGATCTACCCCGAAGGGCATGAA
CTCTGTAATGATCCCTCCGCAGGTTCACCTACGGAGACCTTGTTACGACTTTTACTTCCTCTA

> *Phlyctema* sp. 36-1072f

ATGGGGGGTTCTGGCGAGCATCCACCGGGCATCCAGAGCGAGAGATTTACTACGCTTAGAGCCAGA
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GTTGTAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGAT
TCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGC
CAGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTATTATATAGTACTCAGACGACACTAAATTTC
AGAGTTTGGGGTCCTCTGGCGGGCGCTCACCAGCCGGAGCCGGTGGCCGAGGCGGGCCCGCCAAAG
CAACAAAGGTGTGATGACACGGGTGGGAGATCTACCCCGAAGGGCATGAACTCTGTAATGATCCCT
CCGCAGGTTCACCTACGGAGACCTTGTTACGACTTTTACTTCCTCT

> *Epicoccum* sp. 4-105(2)f

ATCCGAGGTCAAGAGTGTAAAAATGTACTTTTGGACGTCGTCGTTGTGAGTGCAAAGCGCGAGATG
TACTGCGCTCCGAAATCAATACGCCGGCTGCCAATTGTTTTAAGGCGAGTCTACACGCAGAGGCGA
GACAAACACCCAACACCAAGCAGAGCTTGAAGGTACAAATGACGCTCGAACAGGCATGCCCCATGG
AATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTAC
TTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAAC
TATTATGTTTTTTCAGACGCTGATTGCAACTGCAAAGGGTTTGAATGTTGTCCAATCGGCGGGCGG
ACCCGCCGAGGAAACGAAGGTACTCAAAAGACATGGGTAAGAGGTAGCAGACCGAAGTCTACAAAC
TCTAGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCTA

> *Epicoccum* sp. 34-1026(3)f

ATCCGAGGTCAAGAGTGTAAAAATGTACTTTTGGACGTCGTCGTTATGAGTGCAAAGCGCGAGATG
TACTGCGCTCCGAAATCAATACGCCGGCTGCCAATTGTTTTAAGGCGAGTCTGCGCGCGGAGGCGA
GACAAAACACCCAACACCAAGCAGAGCTTGAAGGTACAAATGACGCTCGAACAGGCATGCCCCATG
GAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTA
CTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAA
CTATTAAGTTTTTTCAGACGCTGATTGCAACTGCAAAGGGTTTGAATGTTGTCCAACCGGCGGGCG
GACCCGCCGAGGAAACGAAGGTACTCAAAAGACATGGGTAAGAGGTAGCAGACCGAAGTCCACAAA
CTCTAGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCTA

> *Epicoccum* sp. 34-1029(1)f

AGGTCAAGAGTGTAAAAATGTACTTTTGGACGTCGTCGTTATGAGTGCAAAGCGCGAGATGTACTG
CGCTCCGAAATCAATACGCCGGCTGCCAATTGTTTTAAGGCGAGTCTGCGCGCGGAGGCGAGACAA
AACACCCAACACCAAGCAGAGCTTGAAGGTACAAATGACGCTCGAACAGGCATGCCCCATGGAATA
CCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTAT
CGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATT
AAGTTTTTTCAGACGCTGATTGCAACTGCAAAGGGTTTGAATGTTGTCCAACCGGCGGGCGGACCC
GCCGAGGAAACGAAGGTACTCAAAAGACATGGGTAAGAGGTAGCAGACCGAAGTCCACAAACTCTA
GGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCTA

> *Epicoccum* sp. 35-1049(2)f

ATCCGAGGTCAGAGTGTAAAAATGTACTTTTGGACGTCGTCGTTATGAGTGCAAAGCGCGAGATGT
ACTGCGCTCCGAAATCAATACGCCGGCTGCCAATTGTTTTAAGGCGAGTCTGCGCGCGGAGGCGAG
ACAAAACACCCAACACCAAGCAGAGCTTGAAGGTACAAATGACGCTCGAACAGGCATGCCCCATGG
AATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTAC
TTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAAC

TATTAAGTTTTTTCAGACGCTGATTGCAACTGCAAAGGGTTTGAATGTTGTCCAACCGGCGGGCGG
ACCCGCCGAGGAAACGAAGGTACTCAAAAGACATGGGTAAGAGGTAGCAGACCGAAGTCCACAAAC
TCTAGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCTA

> *Epicoccum* sp. 36-1073(1)f

CTGATCGAGGTCAAGAGTGTAAAAATGTACTTTTGGACGTCGTCGTTATGAGTGCAAAGCGCGAGA
TGTACTGCGCTCCGAAATCAATACGCCGGCTGCCAATTGTTTTAAGGCGAGTCTGCGCGCGGAGGC
GAGACAAAACACCCAACACCAAGCAGAGCTTGAAGGTACAAATGACGCTCGAACAGGCATGCCCCA
TGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAC
TACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGT
AACTATTAAGTTTTTTCAGACGCTGATTGCAACTGCAAAGGGTTTGAATGTTGTCCAACCGGCGGG
CGGACCCGCCGAGGAAACGAAGGTACTCAAAAGACATGGGTAAGAGGTAGCAGACCGAAGTCCACA
AACTCTAGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCTCT
A

> *Penicillium* sp. 6-176f

TGATCCGAGGTCACCTGGATAGATTGATTGGGGGTCGCCGGCGGGCGCCGGCCGGGCCTACGGAGC
GGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGCCCC
CCGGGACCGGGGGGCGGGGCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGG
CATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGC
AATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTT
GAAAGTTTTAACTGATTTAGCTAATCGCTCAGACTGCAATCTTCAGACAGAGTTCAATGGTGTCTT
CGGCGGGCGCGGGCCCGGGGGCAGATGCCCCCCGGCGG

> *Diaporthe* sp. 14-X(2)f

AGGTCAATTTTCAGAAGTTGGGGGTTTAACGGCAGGGCCGCACCAAGACCTTCCAAAGCGAGGGTT
TAACTACTGCGCTCGGGGTCCTGGCGAGCTCGCCACTGAATTTCAGGACCTGCCTTTTCTCAAAGG
CAGTGTCCCATCACCAAGCCAGGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCTCCGGAA
TACCAGAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTT
ATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTC
ATTTTTGTTTTATTCTCAGAGTTTCAGTGTAAAAACAAGAGTTAACTTGGCCGCCGGCGGGCCTGC
TCCTCACCGAGATGGTGAGGGACCCCCCGAGGGGAGCCAGCCTGACGCCGAGGCAACAGTATAGGT
ATAAGTTCACAAAGGGTTGTCTGGAGGTGCGCCGAAGCGCGTTCCAACAATGATCCCTCCGCTGGT
TCACCAACGGAGACCTTGTTACGACTTTTACTTCCTCTA

A3.12 Nonparametric Friedman's two-way test of comparing inhibition effect of endophytic bacteria on the radial growth across three *Neonectria ditissima* strains (ICMP14417, MW15c1 and RS324p).

Total N	51
Test Statistic	3.569a
Degree Of Freedom	2
Asymptotic Sig.(2-sided test)	0.168

a. Multiple comparisons are not performed because the overall test retained the null hypothesis of no differences.

A3.13 Nonparametric Kruskal-Wallis one-way test of radial growth of each *Neonectria ditissima* (ICMP14417, MW15c1 and RS324p) inhibited by endophytic bacteria.

N. ditissima ICMP14417

Total N	51
Test Statistic	49.179[a]
Degree Of Freedom	16
Asymptotic Sig.(2-sided test)	0.000

a. The test statistic is adjusted for ties.

N. ditissima MW15c1

Total N	51
Test Statistic	49.493[a]
Degree Of Freedom	16
Asymptotic Sig.(2-sided test)	0.000

a. The test statistic is adjusted for ties.

N. ditissima RS324p

Total N	51
Test Statistic	48.407[a]
Degree Of Freedom	16
Asymptotic Sig.(2-sided test)	0.000

a. The test statistic is adjusted for ties.

A3.14 One-way ANOVA result of the effect of each endophytic bacterium against *Neonectria ditissima* ICMP14417 when they were pre-inoculated 1, 4 and 7 day(s)**.**

Source	DF	Adj SS	Adj MS	F-Value	P-Value
R1GS-12b -Interval time treatments	2	37.461	18.7303	64.67	0.000
Error	6	1.738	0.2896		
Total	8	39.198			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
42-1206(19)b -Interval time treatments	2	20.7830	10.3915	701.08	0.000
Error	6	0.0889	0.0148		
Total	8	20.8720			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
26-771(23)b -Interval time treatments	2	50.8593	25.4296	642.34	0.000
Error	6	0.2375	0.0396		
Total	8	51.0968			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
41-1182(4)b -Interval time treatments	2	52.687	26.3433	40.65	0.000
Error	6	3.888	0.6480		
Total	8	56.575			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
41-1183(6)b -Interval time treatments	2	63.7188	31.8594	220.60	0.000
Error	6	0.8665	0.1444		
Total	8	64.5854			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
21-606(28)b -Interval time treatments	2	41.3716	20.6858	801.43	0.000
Error	6	0.1549	0.0258		
Total	8	41.5265			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
41-1183(7)b -Interval time treatments	2	104.994	52.4972	167.63	0.000
Error	6	1.879	0.3132		
Total	8	106.873			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
R3L-1b -Interval time treatments	2	36.114	18.057	12.82	0.007
Error	6	8.448	1.408		
Total	8	44.561			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
R3L-6b -Interval time treatments	2	49.586	24.7929	27.11	0.001
Error	6	5.487	0.9145		
Total	8	55.073			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
39-1143(30)b -Interval time treatments	2	81.099	40.5496	64.59	0.000
Error	6	3.767	0.6278		
Total	8	84.866			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
7-208(18)b -Interval time treatments	2	7.424	3.7119	4.77	0.058
Error	6	4.674	0.7790		
Total	8	12.098			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
20-579(18)b -Interval time treatments	2	132.970	66.4848	294.18	0.000
Error	6	1.356	0.2260		
Total	8	134.326			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
26-785(43)b -Interval time treatments	2	134.218	67.1089	69.50	0.000
Error	6	5.794	0.9657		
Total	8	140.012			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
31b1 -Interval time treatments	2	4.542	2.271	2.25	0.187
Error	6	6.060	1.010		
Total	8	10.602			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
31b3 -Interval time treatments	2	13.409	6.7047	19.00	0.003
Error	6	2.117	0.3528		
Total	8	15.526			

Source	DF	Adj SS	Adj MS	F-Value	P-Value
27-801(89)b -Interval time treatments	2	98.73	49.367	23.17	0.002
Error	6	12.78	2.130		
Total	8	111.52			

A3.15 Nonparametric Kruskal-Wallis one-way test of radial growth of *Neonectria ditissima* ICMP14417 inhibited by cell-free filtrate culture of the selected endophytic bacteria.

Total N	228
Test Statistic	195.118[a]
Degree Of Freedom	75
Asymptotic Sig.(2-sided test)	0.000

a. The test statistic is adjusted for ties.

Pairwise comparisons of treatment				
Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.
42-1206(19)T2C2-negative control	-112.333	53.857	-2.086	0.037
R1GS-12bT2C3-negative control	-108.333	53.857	-2.012	0.044
27-801(89)T1C3-negative control	-107.333	53.857	-1.993	0.046

T means culture collection time, C means concentration of the cell-free culture filtrate amended in the agar.

A3.16 One-way ANOVA test of radial growth of *Neonectria ditissima* ICMP14417 inhibited by cell-free filtrate culture of the selected endophytic fungi.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
selected endophytic fungi	18	96.57	5.365	4.11	0.000
Error	40	52.22	1.305		
Total	58	148.79			

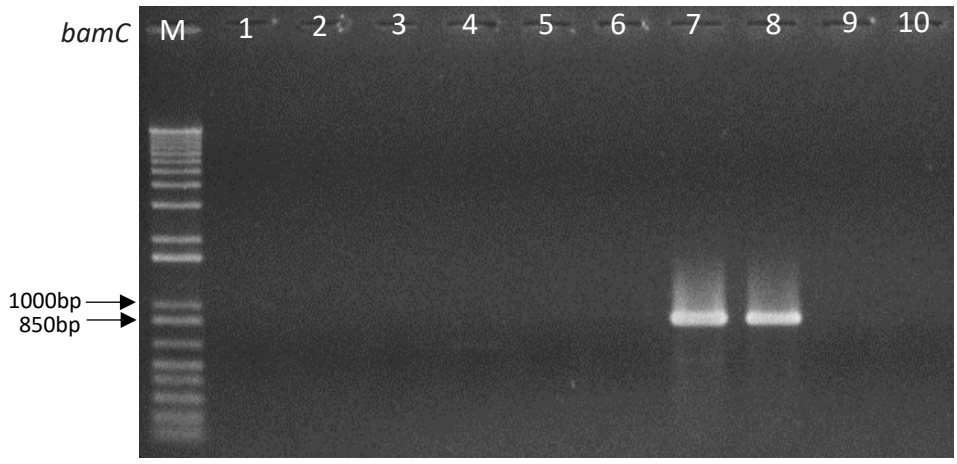
A3.17 One-way ANOVA test of radial growth of *Neonectria ditissima* ICMP14417 inhibited by volatile compounds produced by selected endophytic bacteria.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Selected endophytic bacteria	16	152.49	9.530	7.97	0.000
Error	34	40.66	1.196		
Total	50	193.15			

A3.18 One-way ANOVA test of radial growth of *Neonectria ditissima* ICMP14417 inhibited by volatile compounds produced by selected endophytic fungi.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Selected endophytic fungi	14	22.71	1.6222	2.29	0.037
Error	23	16.27	0.7074		
Total	37	38.98			

A3.19 PCR agarose gel detection and sequences of antibiotics encoding genes



M: 1 kb plus ladder (Invitrogen), 1: negative control (sterile PCR water), 2-10: *Bacillus* spp. 21-606(28)b, 2-771(23)b, R3L-6b, 41-1183(6), 41-1182(4), R1GS-12b, 42-1206(19)b, 41-1183(7)b and R3L-1b, respectively.

> *bamC* R1GS-12b

GTGGGATGGTTCACAAGCCTATATCCGGTTTCACTTCAAATCAAAGCTGATCAGGATATCCCGCAG
CGGATTAAAACAGTGAAAGAAAATTTGCGTCAGATCCCCCAAAAAGGAATAGGTTACGGCCTGATC
AAATATTTGTCCGATCACCCGAAGGCACATGAATGGACAGGACATCCCGAAATACGTTTCAATTAT
TTGGGTCAATTTGATCAAGATGTCCGGAACGGCAAGATGGAGGTATCCCCTTACTCAAGCGGGAAA
ACAGCCAGTGACAACCGTCCCTTGACCTATACACTTGATATCAACGGCATGATTTCAGACGGCCGG
TTGTCGCTGGCTATCAGCTATTGCGGTAAACAATATCAAAGAGAAACAATGGAAGCCTGCGCCGAT
CTCTTAAAAAACAGCCTGCAGCAAGTCATCGCACATTGTGATGCTCAAGATCAAATTCACCTGACG
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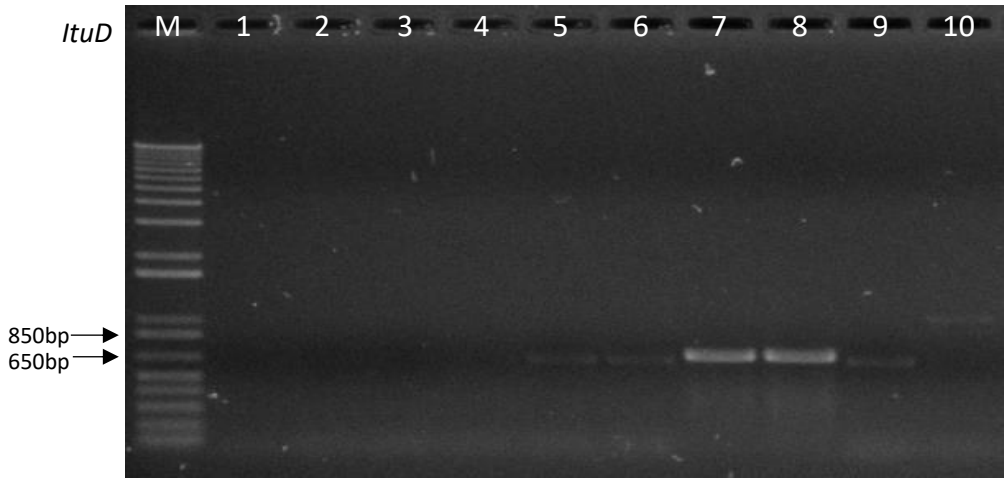

M: 1 kb plus ladder (Invitrogen), 1: negative control (sterile PCR water), 2-10: *Bacillus* spp. 21-606(28)b, 2-771(23)b, R3L-6b, 41-1183(6), 41-1182(4), R1GS-12b, 42-1206(19)b, 41-1183(7)b and R3L-1b, respectively.

> *ItuD* R1GS-12b

GAATTGACAAAGACAATGAACGCGCAGCGCGCTATTTTAACGGTCAGTGTTATTGCTTTTCAAGTG
TATATGCGGGAAATAGGGGTGAAGCCCCGCTTCCTGGCAGGCCATAGCTTAGGCGAATATTCAGCG
CTTGTCTGTGCCGGCGCCCTTTCTTTTCAGGATGCCGTTACCCTTGTAAGGGAGCGGGGAATTCTT
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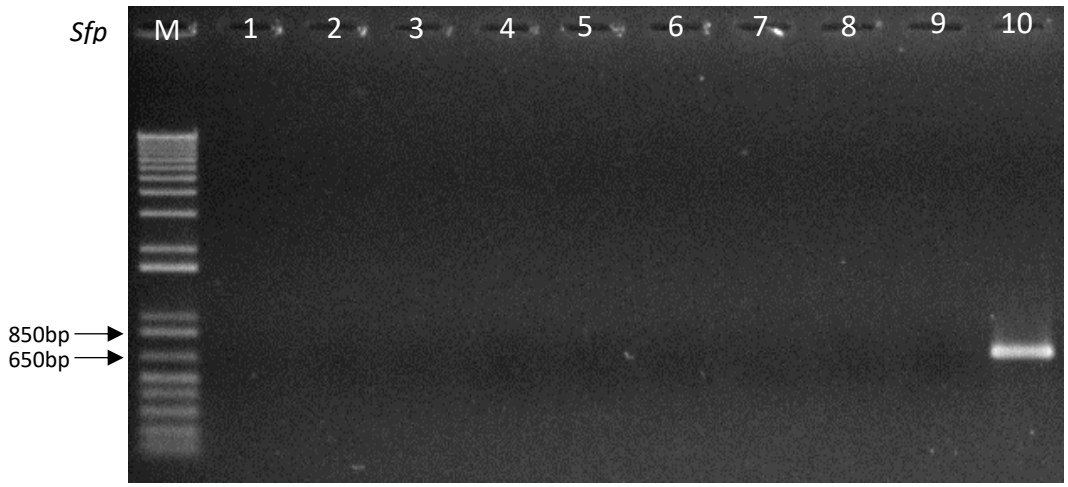
AACGTTGCAGGAAATAT



M: 1 kb plus ladder (Invitrogen), 1: negative control (sterile PCR water), 2-10: *Bacillus* spp. 21-606(28)b, 2-771(23)b, R3L-6b, 41-1183(6), 41-1182(4), R1GS-12b, 42-1206(19)b, 41-1183(7)b and R3L-1b, respectively.

> *sfp* R3L-1b

TCATGACTTTCATATCACCTGAAAAACGGGAGAAATGCCGGAGGTTTTATCATAAAGAAGATGCTC
ACCGCACCCTGCTGGGAGATGTGCTCGTTCGCTCAGTCATAAGCAGGCAGTATCAGTTGGACAAAT
CCGATATCCGCTTTAGCACGCAGGAATACGGGAAGCCGTGCATCCCTGATCTTCCCGACGCTCATT
TCAACATTTCCCACTCCGGCCGCTGGGTCATTGGTGCGTTTGATTCACAGCCGATCGGCATAGATA
TCGAAAAAACGAAACCGATCAGCCTTGAGATCGCCAAGCGCTTCTTTTC

Appendix for Chapter 4

A4.1 One-way ANOVA test of number of apple stem pieces positive for background endophytic bacterial colonies growing on nutrient agar (NA) amended with four antibiotics (streptomycin, erythromycin, chloramphenicol and rifampicin) at three concentrations (50 ppm, 75 ppm and 100 ppm).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
NA + Antibiotics	12	25.69	2.1410	2.98	0.010
Error	26	18.67	0.7179		
Total	38	44.36			

A4.2 Nonparametric Kruskal-Wallis one-way test of radial growth of each *Neonectria ditissima* isolate (ICMP14417, MW15c1 and RS324p) inhibited by wild type and mutant bacterial strains of 42-1206(19)b, 21-606(28)b, R3L-6b, 7-208(18)b, 20-579(18)b and 31b3.

N. ditissima ICMP14417

Total N	40
Test Statistic	38.506[a]
Degree Of Freedom	12
Asymptotic Sig.(2-sided test)	0.000

a. The test statistic is adjusted for ties.

N. ditissima MW15c1

Total N	40
Test Statistic	37.968[a]
Degree Of Freedom	12
Asymptotic Sig.(2-sided test)	0.000

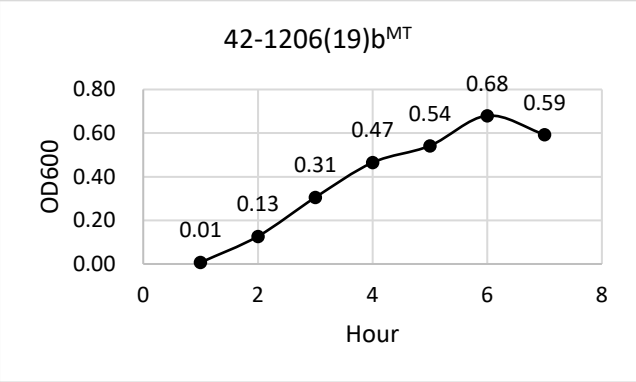
a. The test statistic is adjusted for ties.

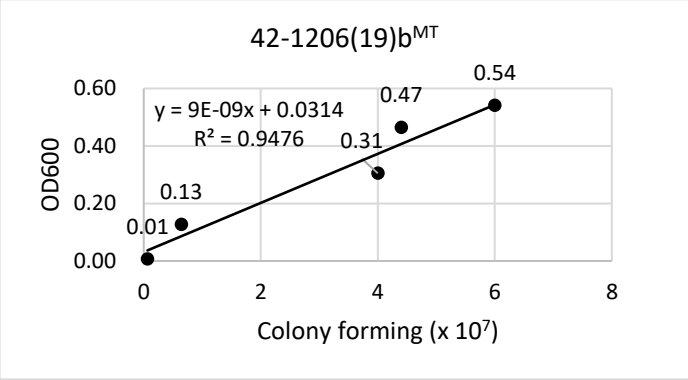
N. ditissima RS324p

Total N	40
Test Statistic	37.383[a]
Degree Of Freedom	12
Asymptotic Sig.(2-sided test)	0.000

a. The test statistic is adjusted for ties.

A4.3 Standard growth curve of *Bacillus* sp. 42-1206(19)b mutant strain resistant to 125ppm rifampicin (42-1206(19)b^{MT}). (a) Growth curve based on OD600; (b) Standard growth regression line based on OD600 and CFU/mL.

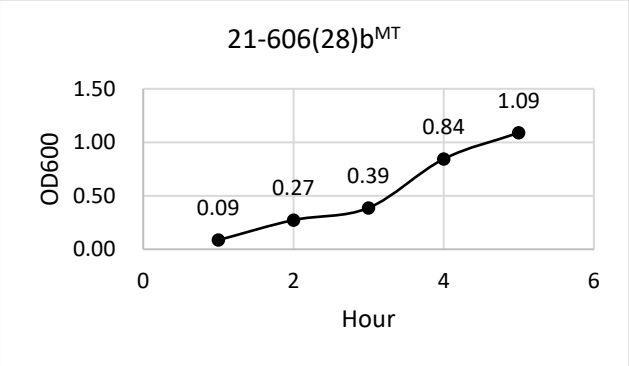


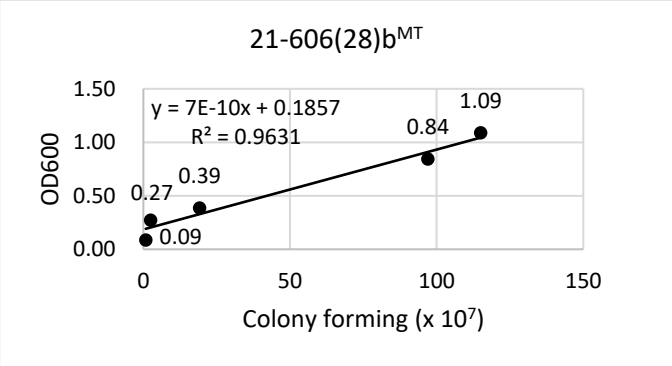


42-1206(19)b^{MT}	CFU/mL	OD600
2 h	640000	0.01
4 h	6400000	0.13
6 h	40000000	0.31
8 h	44000000	0.47
10 h	60000000	0.54
12 h	46000000	0.68
15 h	-	0.59

- means no data.

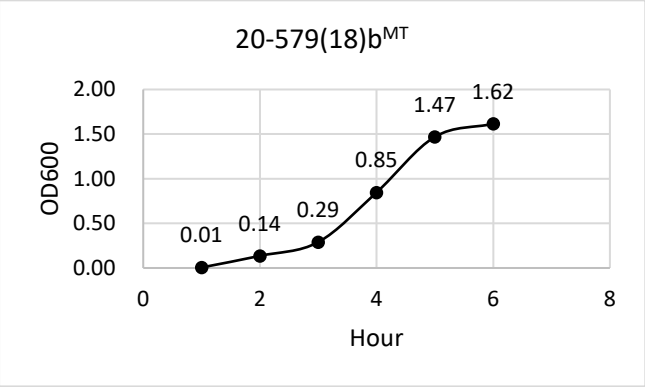
A4.4 Standard growth curve of *Pseudomonas* sp. 21-606(28)b mutant strain resistant to 125ppm rifampicin (21-606(28)b^{MT}). (a) Growth curve based on OD600; (b) Standard growth regression line based on OD600 and CFU/mL.

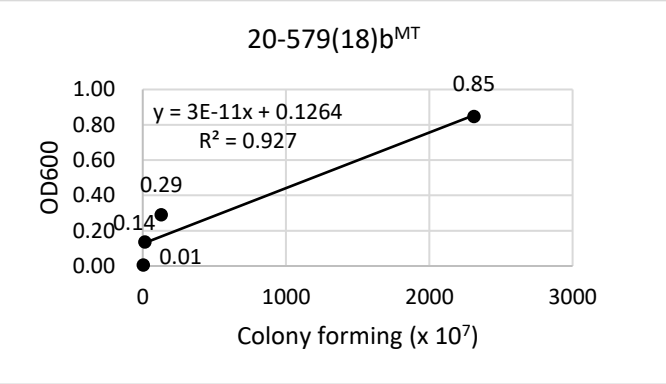




21-606(28)b^{MT}	CFU/mL	OD600
2 h	8200000	0.09
4 h	25000000	0.27
6 h	192000000	0.39
10 h	970000000	0.84
12 h	1150000000	1.09

A4.5 Standard growth curve of *Pseudomonas* sp. 20-579(18)b mutant strain resistant to 125ppm rifampicin (20-579(18)b^{MT}). (a) Growth curve based on OD600; (b) Standard growth regression line based on OD600 and CFU/mL.

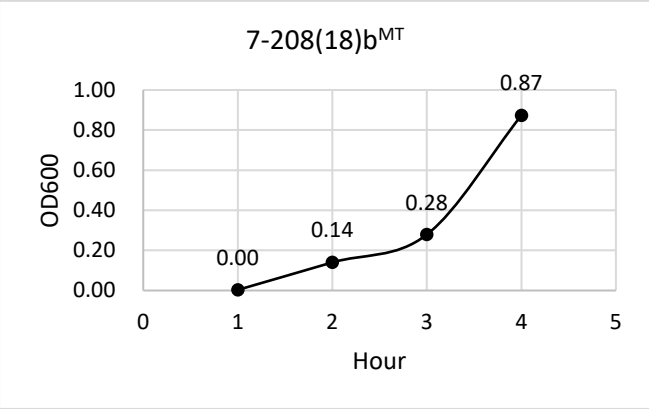


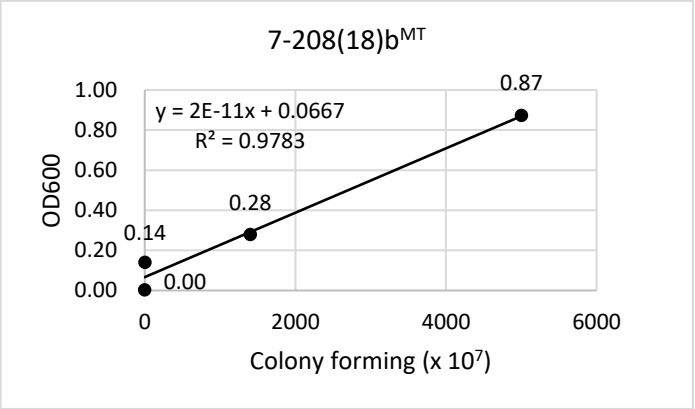


20-579(18)b^{MT}	CFU/mL	OD600
2 h	34000000	0.01
4 h	149000000	0.14
6 h	1280000000	0.29
8 h	2.31E+10	0.85
10 h	-	1.47
12 h	-	1.62

- means no data.

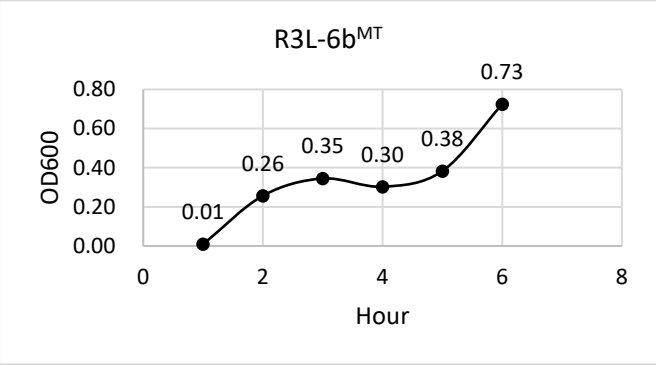
A4.6 Standard growth curve of *Pseudomonas* sp. 7-208(18)b mutant strain resistant to 125ppm rifampicin (7-208(18)b^{MT}). (a) Growth curve based on OD600; (b) Standard growth regression line based on OD600 and CFU/mL.

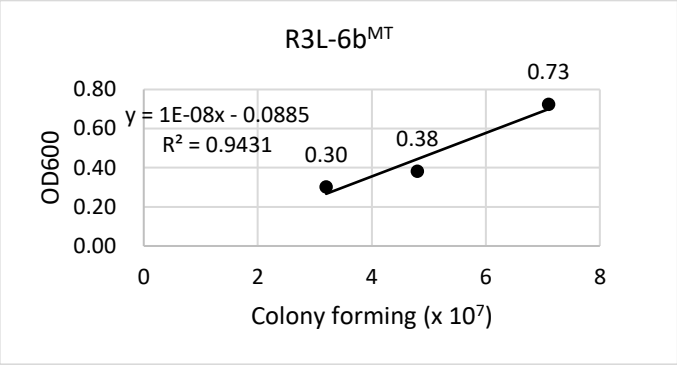




7-208(18)b^{MT}	CFU/mL	OD600
2 h	3700000	0.00
4 h	26800000	0.14
6 h	1.4E+10	0.28
8 h	5E+10	0.87

A4.7 Standard growth curve of *Pseudomonas* sp. R3L-6b mutant strain resistant to 125ppm rifampicin (R3L-6b^{MT}). (a) Growth curve based on OD600; (b) Standard growth regression line based on OD600 and CFU/mL.

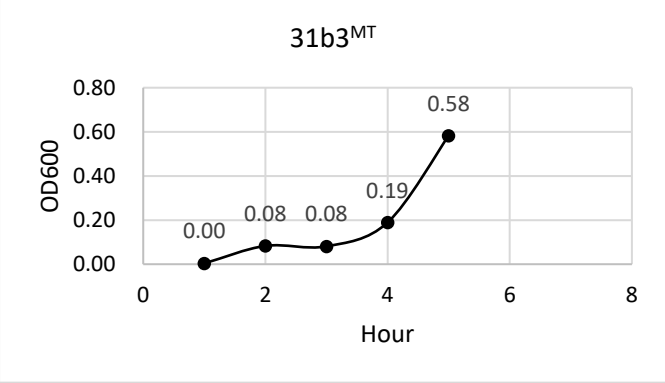


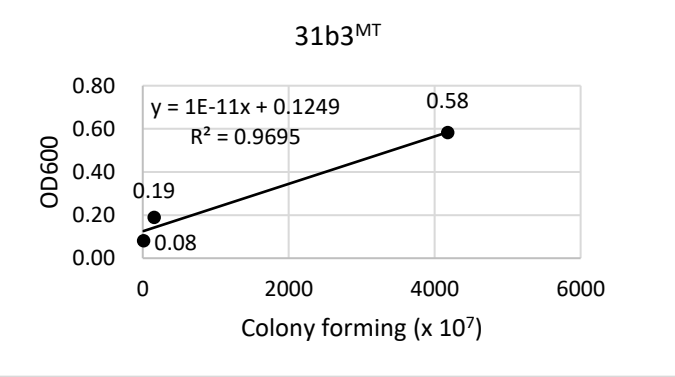


R3L-6b^{MT}	CFU/mL	OD600
2 h	-	0.01
4 h	17400000	0.26
6 h	56000000	0.35
10 h	32000000	0.30
12 h	48000000	0.38
15 h	71000000	0.73

- means no data.

A4.8 Standard growth curve of *Pseudomonas* sp. 31b3 mutant strain resistant to 125ppm rifampicin (31b3^{MT}). (a) Growth curve based on OD600; (b) Standard growth regression line based on OD600 and CFU/mL.





31b3^{MT}	CFU/mL	OD600
2 h	14900000	0.00
4 h	51000000	0.08
6 h	133000000	0.08
8 h	1560000000	0.19
10 h	4.18E+10	0.58

Appendix for Chapter 5

A5.1 One-way ANOVA test of population of rifampicin-resistant bacterial mutants (log_{10} CFU/cm stem) recovered from the inoculation point of detached apple shoots (four replicates) inoculated with the three tested bacterial mutants assessed 28 days after inoculation with *Neonectria ditissima* (+) or 0.005% Tween 20 as untreated control (-).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	5	3.439	0.6879	2.19	0.104
Error	17	5.346	0.3145		
Total	22	8.786			

A5.2 One-way ANOVA test of population of rifampicin-resistant bacteria (log_{10} CFU/ half of 1 cm stem section) recovered from half of the 1 cm stem section at the inoculation point of attached apple shoots inoculated with two *Pseudomonas* sp. mutant strains at the 16 weeks assessment. Shoots were inoculated with endophytic *Pseudomonas* sp. mutant strains followed 14 days later with *Neonectria ditissima*/0.005% Tween 20 or *N. ditissima*/0.005% Tween 20 followed 14 days later with endophytic *Pseudomonas* sp. mutant strains.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	7	1.756	0.2508	0.46	0.846
Error	13	7.078	0.5444		
Total	20	8.833			

A5.3 One-way ANOVA test of lesion length on the shoots with *Neonectria ditissima* inoculated measured at the 8 weeks shoot harvesting time. Shoots were inoculated with endophytic *Pseudomonas* sp. mutant strains/PBS followed 14 days later with *N. ditissima*/Tween 20 or *N. ditissima*/Tween 20 followed 14 days later with endophytic *Pseudomonas* sp. mutant strains/PBS.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	5	296.6	59.32	3.03	0.026
Error	28	548.3	19.58		
Total	33	844.9			

A5.4 One-way ANOVA test of lesion length on the shoots with *Neonectria ditissima* inoculated measured at the 16 weeks shoot harvesting time. Shoots were inoculated with endophytic *Pseudomonas* sp. mutant strains/PBS followed 14 days later with *N. ditissima*/Tween 20 or *N. ditissima*/Tween 20 followed 14 days later with endophytic *Pseudomonas* sp. mutant strains/PBS.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	5	0.04089	0.008178	0.44	0.819
Error	27	0.50602	0.018742		
Total	32	0.54692			